

**FREQUENCY OF CCR2V64I AND CCR5 Δ 32 HOST GENES AND THEIR
ASSOCIATION WITH HIV INFECTION AMONG PREGNANT WOMEN FROM
HARARE, ZIMBABWE**

WHITE SOKO

A mini-thesis submitted in partial fulfilment of the requirements for
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Key words

HIV in pregnant women

Host genetic factors

Gene polymorphism

Mutation

Protective effect

CCR5-Δ32

CCR2V64I

Vaccines

Chemotherapeutic drugs

Zimbabwe



Abbreviations

AIDS-Acquired Immunodeficiency Syndrome

HIV-Human Immune Deficiency Virus

CCR2 –Cystein-Cystein Linked Chemokine Co-receptor 2

CXCR4- Cystein-X-Cystein Linked Chemokine Co-receptor 4

CCR5- Cystein-Cystein Linked Chemokine Co-receptor 5

CCR5 Δ 32- Cystein-Cystein Linked Chemokine Co-receptor 5 delta 32

DNA-Deoxyribonucleic Acid

UNAIDS-United Nations programme on HIV disease and Aids

MgCl₂ –Magnesium Chloride

FRET-Flourescent Resonance Energy Transfer

min-Minutes

NSI-Non-syncytial inducing

SI-Syncytial inducing

STIs – Sexual Transmitted Infections

HSV-2- Herpes Simplex Virus-2

gp- glycoprotein

PCR-Polymerase Chain Reaction



μl – Microlitre

ml- Millilitre

S-Seconds

SDF-1 -Stromal derived factor-1

SSA- Sub Sahara Africa



DEDICATION

I dedicate this thesis to my late beloved father Enosi Soko who died just when I was developing the proposal for this thesis. His fatherly encouragement was a source of inspiration to me and will be missed forever.



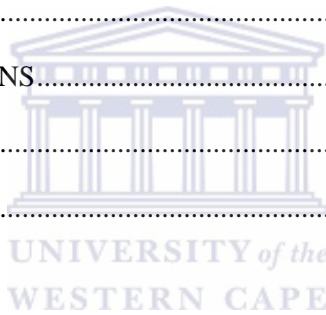
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ABSTRACT

Background: HIV/AIDS has become a major public health problem, with host genetic factors such as CCR2-V641 and CCR5-Δ32 genes determining the probability of acquiring it and the rate of disease progression. The frequency of these genes is not well described in high HIV endemic areas like Zimbabwe among pregnant women. Yet this information is important in the design and development of HIV drugs and vaccines.

Aim: To determine and compare the prevalence of CCR5-Δ32 and CCRV64I genes in HIV positive and HIV negative population of pregnant women from Harare, in Zimbabwe.

Design: A cross-sectional study of CCR5- Δ32 and CCR2V641 chemokine co-receptors in 76 HIV positive and 166 HIV negative populations of pregnant women.

Procedures: DNA was extracted from sera samples using the Boom and co-workers (1990) method. SYBR green technology and melting curve analyses were carried out on an Applied Biosystems Step One™ Plus Real-time PCR machine while FRET technology and a Light Cycle™ technology real-time PCR machine were used to determine the frequency of CCR5-Δ32 and CCR2V641 respectively.

Data analysis: Data was entered and analysed using a Statistical Package for Social Sciences (SPSS) version 16 (SPSS, IL, USA). Chi-square (χ^2) tests and relative risks (RR) for CCR5-Δ32 and CCR2V641 chemokine co-receptors were used to assess if there was any association between these host genes and HIV infection. Univariate analysis was also used. Notably multivariate regression analysis was used to control for confounders.

Results: The proportion of pregnant women with the homozygous CCR2V64I gene was 24.38% and this gene was two times more associated with HIV infection than in those without it (RR= 2.32, 95% CI-1.38-3.92). No CCR5-Δ32 deletion was detected in the studied population.

Conclusion: The homozygous CCR2V64I gene and STIs were more prevalent in HIV infected pregnant women than in uninfected pregnant women and no homozygous CCR5-Δ32 gene was detected in this study.

CHAPTER 1: INTRODUCTION

1.1 Overview of the HIV epidemic

Human Immunodeficiency Virus type 1 (HIV-1) continues to be a significant cause of mortality and morbidity in the world (Kaul, Makadzange & Rowland-Jones, 2000), with a disproportionate transmission and distribution among different regions. According to UNAIDS and the World Health Organization (2008b), in 2007 close to 1.5 million pregnant women were infected. Such pregnancies in HIV infection has resulted in over 250 000 children dying from the epidemic, leaving two million of them living with the HIV infection (UNAIDS, 2008b). In Africa, the HIV epidemic has been heterogeneously distributed, with Sub-Saharan Africa (SSA) being one of the most heavily affected regions. The region is host to five of the leading countries in HIV infection in the world. Women in this region constitute close to 60% of the total number of infected people (UNAIDS, 2008c). Because of this the number of infected children is expected to increase.

In Zimbabwe HIV/AIDS remains a major public health problem as the country is still rated among the top five countries hardest hit by the epidemic in the world (UNAIDS, 2008a).

According to UNAIDS (2008a), close to 15 % of the total population of 14 million was infected with the HIV/AIDS in 2007 and 52 000 of them being pregnant women living with HIV. By 2007 close to 120 000 children were living with HIV in Zimbabwe (UNAIDS, 2008a), most of whom became infected through mother-to-child transmission.

Although there has been a disproportionate transmission and distribution of HIV epidemic in the world, with alarming rates in SSA, currently there is very little explanation for this observation (Martinsoni, Chapman, Rees, Liu & Clegg, 1997). Martinsoni and co-workers (1997) suggest that the distribution pattern of the rate and severity of the HIV epidemic may

be indicative of greater underlying susceptibility to the virus in certain populations, which may be attributable to certain ubiquitous factors such as host genetics.

Sullivan and collaborators (2001) note that certain host genetic factors could have an impact on the heterosexual transmission of HIV epidemics. This observation has been documented by Rowland-Jones (1998) who notes that despite multiple sexual exposures to HIV-1, some individuals remained HIV negative. On the other hand the discovery of long-term non-progressors (LTNP) in HIV infection provides a unique opportunity for further investigations into the role of host genetic factors in the progression of HIV infection to AIDS. The mechanism underlying this resistance to HIV infection remains unclear, although multiple factors have been hypothesized to be responsible for the pathogenesis of the HIV.

Mounting evidence now suggest that HIV-1 infects the target cells using the CD4 cells receptor in conjunction with co-receptor molecules. Among the various chemokine co-receptors that support virus entry are the chemokine co-receptor 5 (CCR5) and the CX chemokine co-receptor 4 (CXCR4). These have been identified as the major co-receptor of macrophage-tropic non-syncytium inducing (NSI) and T-cell tropic syncytium-inducing (SI) primary isolates, respectively (Ometto, Zanchetta, Mainardi, Salvo, Garcia-Rodriguez & Gray, 2000). It has been further demonstrated that the majority of NSI isolates utilize only CCR5, whereas most SI isolates, besides CXCR4, also use additional co-receptors like CCR2 among others to enter target cells (Ometto *et al.*, 2000).

Mutations in CCR5 and CXCR4 have either influenced the probability of acquiring HIV infection or have affected the rate of disease progression, in infected adults (Kostrikis, Neumann, Thomson, Korber, McHardy & Karanicolas, 1999) or infants (Misrahi, Teglas, Burgard, Mayaux, Rouzioux & Delfraissy, 1998). A 32-nucleotide deletion in CCR5, the CCR5- Δ 32 allele, leads to the translation of a defective CCR5 protein. This protein has been

identified as a natural genetic polymorphism reducing the risk of acquiring HIV-1 infection and the rate of disease progression in infected adults (Kostrikis *et al.*, 1999; Misrahi *et al.*, 1998). The effect of CCR5-Δ32 gene on HIV transmission is dependent on the allelic composition of the gene.

Similarly, the CCR2 V64I mutation, a conservative valine to isoleucine substitution in the transmembrane region, has been observed as having some protective effects against HIV infection and reduce progression of HIV to AIDS (Chakraborti & Banerjee, 2002). The protective effect of CCR2 V64I is believed to involve cross regulation through linkage disequilibrium, particularly in the regulatory region or promoter region of CCR5. CCR2V64I is said to be more pronounced among Africans (Matte & Roger, 2001), yet this is the most affected race (UNAIDS, 2008c) by the HIV pandemic than the non-African race, making the protective abilities of this chemokine co-receptor questionable and controversial.

1.2 Background

In mid 1996, scientists made a breakthrough in the field of HIV research when they discovered that chemokine co-receptors were crucial in the transmission of HIV-1 infection (Cohn & Weaver, 2006). That finding boosted the prospects of finding a therapy for HIV infection. Later on this finding was followed by yet another interesting discovery that some people remained uninfected by HIV-1, despite multiple unprotected sexual exposures while others when infected with HIV-1 remained asymptomatic for a long time and these people were deemed as long-term non-progressors (Rowland-Jones, 1998). Both developments were attributed to the role of chemokine co-receptors in HIV infection (Kostrikis *et al.*, 1999; Misrahi *et al.*, 1998; Rowland-Jones, 1998).

Before the discovery of chemokines co-receptors, only the role of the CD4 molecule as a high-affinity receptor for HIV was known; but it was later on discovered that the CD4 molecule receptor was not sufficient for HIV infection to occur (Stephens, Claiborne, Reich, David, Goldstein & David, 1998). It was in 1996 that the identity and contribution of other co-receptors in HIV infection and disease pathogenesis were recognized (Stephens *et al.*, 1998).

Chemokine receptors are inherited host genetic factors that are known to determine the structural, physiological and biochemical characteristics of a host's cell. This inherited information can be passed on from one generation to the other (Tylor, Green & Stout, 1997). In certain circumstances, chemokines receptor genes undergo natural mutations or mutate under the influence of mutagens.

Factors leading to the concurrence of particular mutations include selective pressure, and historical events such as disease epidemics. While the cause of increasing HIV prevalence rates in parts of Asian and African continents may be attributed to social and demographic factors, as well as differences in the phenotype of the circulating viruses (Soto-Ramirez, Benjito, MacLane, Marlink, O' Hara & Suttent, 1996), the racial distribution of risk factors to HIV infections has not been adequately explained. This raises the possibility that differences in the distribution of host genetic factors might influence the rate of transmission or the speed at which the epidemic is spreading in different racial groups. Infectious diseases such as the bubonic plague, small pox and pathogens including *Yersinia pestis* (an enteric pathogen) have been postulated to have generated a 32 base deletion in the CCR5 gene to CCR5-Δ32 that is believed to account for the variations in prevalence of particular mutations within specific populations (Lalani, Masters, Zeng, Barret, Pannu, Everette *et al.*, 1999; Libert, Cochaux, Beckman, Samson, Aksenova, Cao *et al.*, 1998). Therefore, differences in prevalence of

certain mutations are increasingly pointing to differences in genetic polymorphism as an important factor that may be responsible for the distribution pattern and spread of HIV in the world (Michael, Nelson, KewalRamani, Chang, O'Brien, Mascola *et al.*, 1998). Despite that observation, prevalence rate and role of these factors in HIV transmission in high HIV endemic areas like Zimbabwe are not well described.

1.3 Problem statement

Although there are indications that CCR5- Δ 32 and CCR2V641 host genes confer some protection against HIV infection (Kostrikis *et al.*, 1999; Misrahi *et al.*, 1998; Christine, Hogan, Scott & Hammer, 2001; Anzala, Ball, Rostron, O'Brien, Plummer & Rowland-Jones, 1998), in Zimbabwe the role of these chemokine receptors in the transmission of HIV is not well described (Williamson, Loubser, Brice, Joubert, Smith, Thomson *et al.*, 2000). Yet the country is rated among the top five countries hardest hit by the pandemic in the world. It is therefore of great significance to understand the host genetic factors that are involved in the transmission of HIV, so that appropriate intervention programs can be put in place.

1.4 Purpose of the study

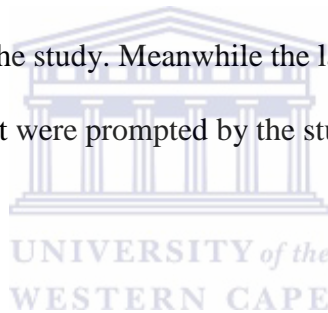
The proposed study intends to produce information on the key host genes that are associated with HIV transmission in the African population of pregnant women in Harare. Furthermore the information that is going to be produced might contribute to the understanding of the role of CCR5- Δ 32 and CCR2V641 host genes, in the dynamics, evolution of the epidemic, and in designing effective chemotherapeutic drugs and vaccines directed against the chemokine co-receptor sites.

1.5 Outline of the thesis

This thesis describes how the prevalence of CCR5- Δ 32 and CCR2V641 genes were determined and compared in HIV infected and uninfected population of pregnant women

from Harare in Zimbabwe. Chapter 2 reviews the literature on the current global, regional and the Zimbabwean HIV situation. It explains how CCR5-Δ32 and CCR2V641 genes could have influenced this. The literature review chapter further deal with the global distribution of CCR5-Δ32 and CCR2V641 genes, and on how the discovery of these chemokine co-receptors have contributed in the designing of effective HIV-1 chemotherapeutic drugs and vaccines directed against the chemokine co-receptor sites.

Chapter 3 describes different methods that were systematically employed in order to achieve the aim of the study. This chapter will be then followed by Chapter 4 which tells us about the findings (results) of this study. Chapter 5 which is the discussion section describes the results of the study and relates them to other studies elsewhere. Chapter 6 then highlights the conclusion that was drawn from the study. Meanwhile the last chapter which is Chapter 7 outlines the recommendations that were prompted by the study findings.



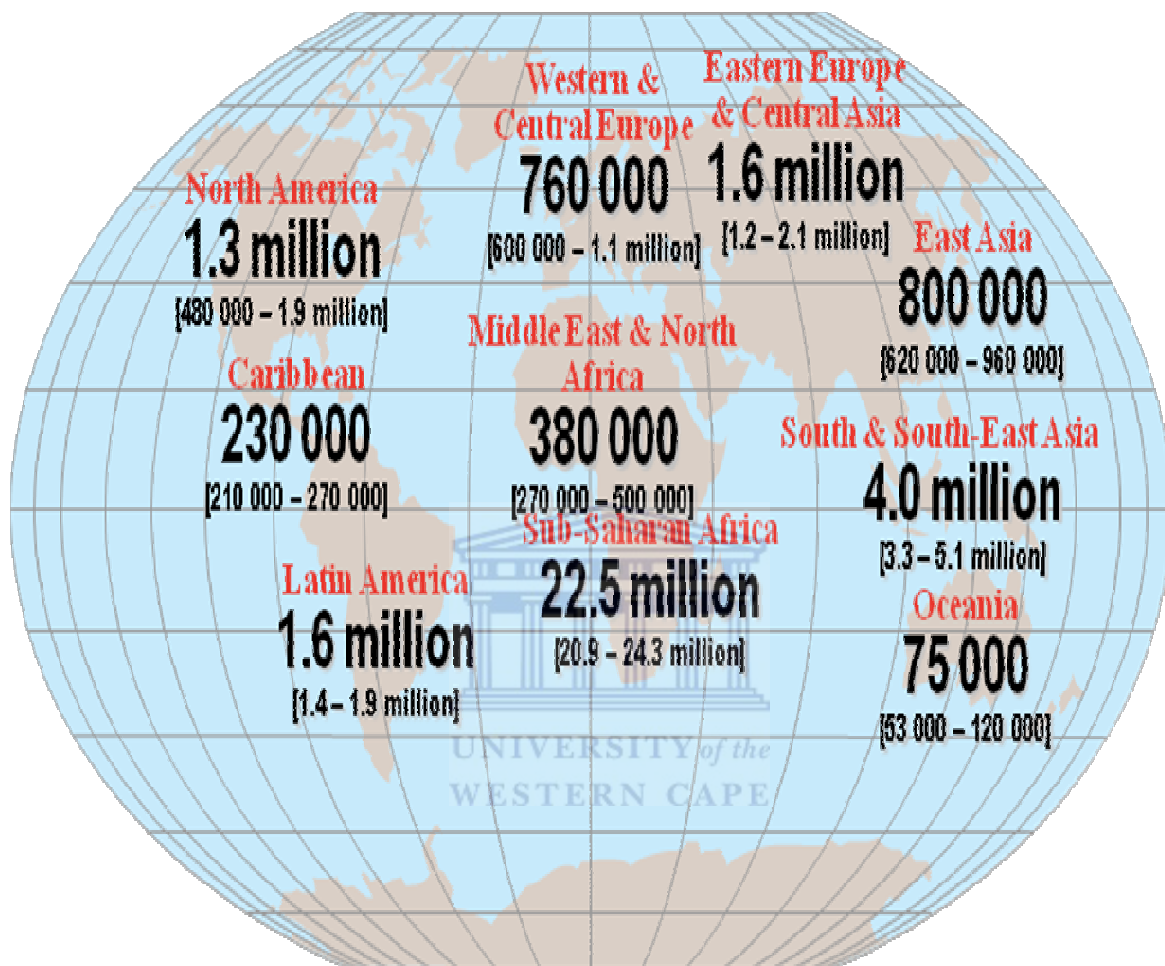
CHAPTER 2: LITERATURE REVIEW

2.1 Outline of the literature review

The role of host genes in HIV transmission has attracted a lot of scientific interest and as a result a lot of literature is currently being generated. The current study investigates the frequency and role of host genes in HIV infection in Zimbabwe among pregnant women. The literature herein describes the global epidemiology of HIV, global distribution of host genes and how these host genes may have influenced the global distribution and transmission of HIV infection. The literature also explains how the discovery of these chemokines co-receptors have contributed to the designing and development of HIV-1 chemotherapeutic drugs and vaccines directed against the chemokine co-receptor sites.

2.2 Epidemiology of HIV

The global distribution of the HIV infection has been very interesting. The first major epidemics of HIV were described in countries of central and eastern Africa, but today the epidemic is now far worse in the southern part of the continent, the Sub Sahara Africa (SSA). SSA is only home to 10% of the world's population, yet today more than 70% (22.5 million) of people with HIV infection in the world is currently found in this region (UNAIDS, 2007) as shown in Figure 1.



Total: 33.2 million

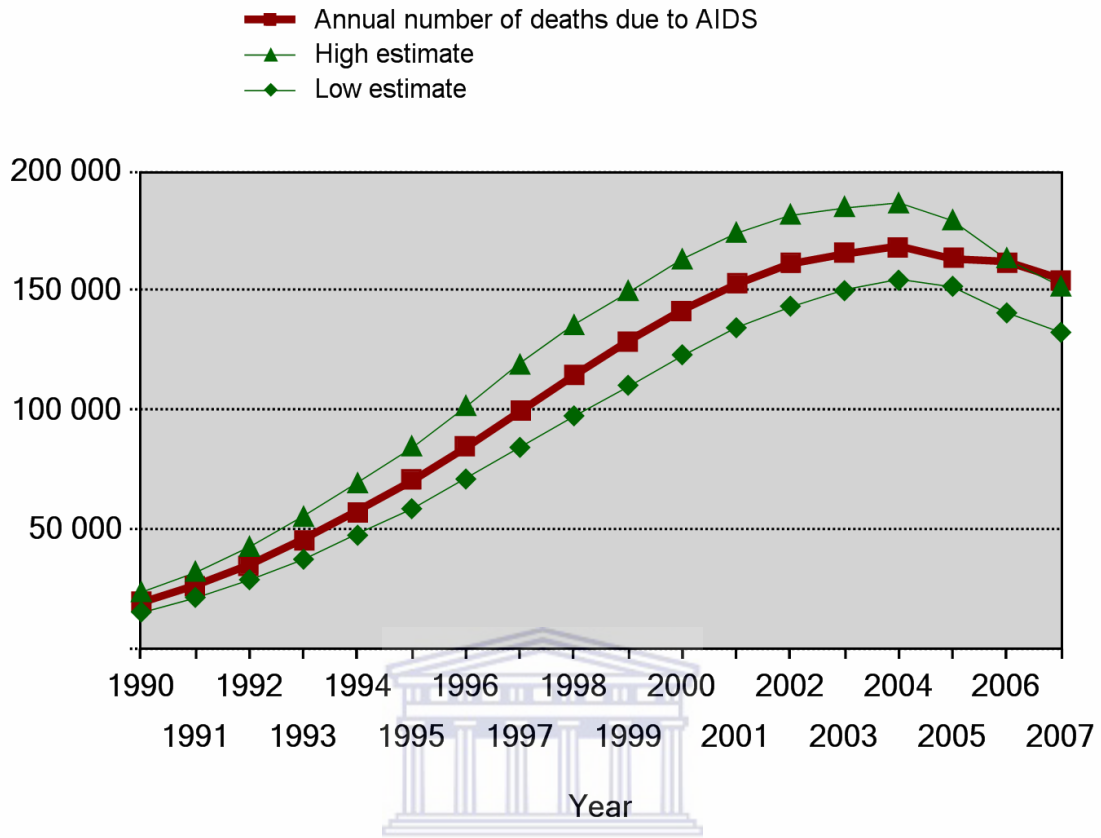
Source: UNAIDS, 2008b

Figure 1 : Global estimates of HIV infected adults and children as end of 2007.

This global picture is said to be the result of distinct epidemiological profile resulting from a variety of contributing factors displaying large heterogeneity in different regions. These factors include: frequency of sexual contact, number and risk characteristics of sexual partners, concurrent partners, condom usage, circumcision, alcohol and/or drug use, stage of the epidemic and likelihood of encountering infectious individuals, background immunological status, pressure from co-infections, access to relevant health care and host genetic factors (UNAIDS, 2007; Libert *et al.*, 1998).

A number of things have also happened in the epidemiology of HIV since the discovery of the interplay between host and viral factors. A breakthrough in the understanding of host factors (chemokine co-receptors) that affect disease progression and susceptibility to HIV infection occurred in 1996, when it was observed that chemokine co-receptors were necessary for HIV entry into CD4 cells (Christine *et al.*, 2001). This discovery was followed by a decline in HIV prevalence globally, regionally and Zimbabwe was no exception. For instance the global annual number of new HIV infections declined from 3.0 million in 2001 to 2.7 million in 2007 (UNAIDS, 2008b). In SSA most countries recorded a decline in HIV infection among the pregnant women aged between 15-49 years between 2002 and 2006 (UNAIDS, 2008c). In Zimbabwe HIV prevalence in pregnant women declined from 28% to 16 % between 2002 to 2006 (UNAIDS, 2008a). These changes in HIV prevalence also resulted in the drop in Zimbabwe's annual HIV death rates between 2004 and 2007 as shown in Figure 2.

Estimated number of deaths due to AIDS 1990-2007



Source: UNAIDS, 2008a

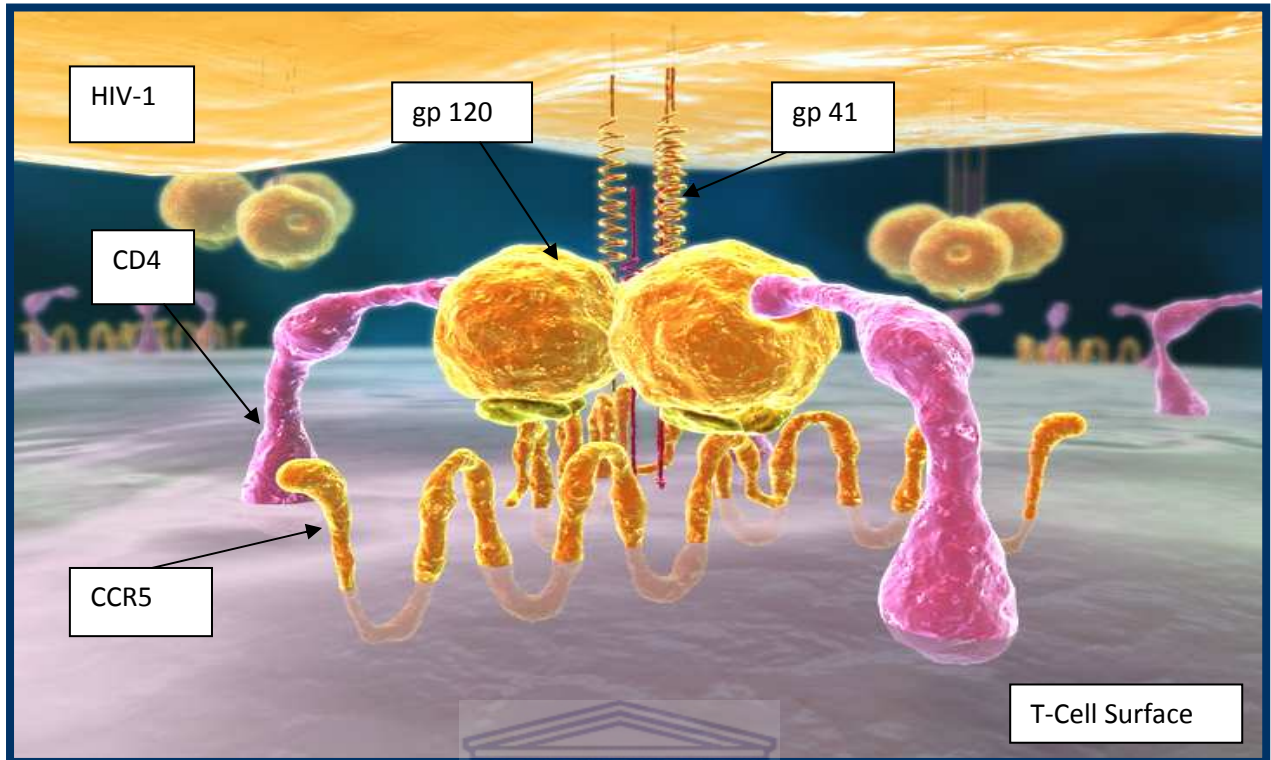
Figure 2: The annual number of deaths in Zimbabwe due to AIDS between 1990 and 2007

This decline in HIV death rate between 2004 and 2007 (Figure 2) has been mainly attributed to sexual behaviour change and condom use (UNAIDS, 2005; UNAIDS, 2008a), and very little credit has been given to the influence of host genetic factors in HIV-1 infection. Yet the host genetic factors seem to play a crucial role in HIV infection and disease progression, in turn affecting the global trend and distribution of HIV infection.

2.3 HIV infection and progression to AIDS

Entry into target cells by HIV occurs by a multi-step process that culminates with the fusion of viral and cellular membrane (Figure 3). To enter the target cells, HIV interacts with CD4 receptor via its gp120 protein, thereby stimulating a conformational change in gp120, which exposes a portion of transmembrane glycoprotein gp41, and allows access of the gp120 V-loop to either CCR5 or CXCR4. Subsequently, a peptide in gp41 causes the fusion of the viral capsid to enter the target cell. (Buseyne, Janvier, Teglas, Ivanoff, Burgard & Bui, 1998; Pierson & Doms, 2003) (Figure 3). HIV-1 infection depends on the chemokine receptors and co-receptors for attachment to the hosts' cells. Polymorphisms in these human chemokine receptor systems will therefore affect the evolution of HIV-1 (Kaslow & McNicholl, 1999).





(Figure adapted from Michael Zaiac, New Product Development, 2005)

Figure 3: CCR5 co-receptor conformational changes exposes the co-receptor binding site in gp120. HIV-1 enters the target cells after interacting with CD4 receptor via its gp120 protein.

2.3.1 Stages in HIV infection

Following infection (Figure 4), an acute disease syndrome may occur within two to five weeks characterised by general lymphopenia and high-level viremia. Clinical manifestations of primary stage are characterised by general malaise, headache, myalgia, arthralgia, diarrhea and lymphadenopathy (Perelson, Essenger & Ho, 1997; Fauci, 1998). An acute immune response involving viral neutralising antibodies and cytolytic T-cells reduces the initial viremia substantially, and a baseline level of viral replication is established and this is referred to as the viral set-point. HIV result in the progressive depletion of cells of the immune system: CD4 T-lymphocytes, macrophages, monocytes, and dendritic cells. T-cell

depletion may be as a result of direct killing of mature T-cells with the rapid replication of HIV-1 (Perelson, Essunger & Ho, 1997). Alternatively CD4 cells may be lost by activation induced death, innocent by-stander effect or by lack of proper cytokine stimulation (Clark, de Boer, Wolthers & Miedema, 1999; Ho, Neumann, Perelson, Chen, Leonard & Markowitz, 1995). The amount of virus in the blood appears to be well correlated with disease development and progression. Eventually, the weakened immune system becomes unable to control a range of endogenous and environmental pathogens, leading to clinical illness and, ultimately death in most individuals (Fauci, 1998; Kahn & Walker, 1998). However, certain precipitating factors can either hurry or delay the disease progression process. These comprise of host genetic factors like CCR5-Δ32 and CCR2V64I mutations that have been observed to delay progression of HIV infection to AIDS or death (Christine, Hogan, Scott, Hammer, 2001) among others.



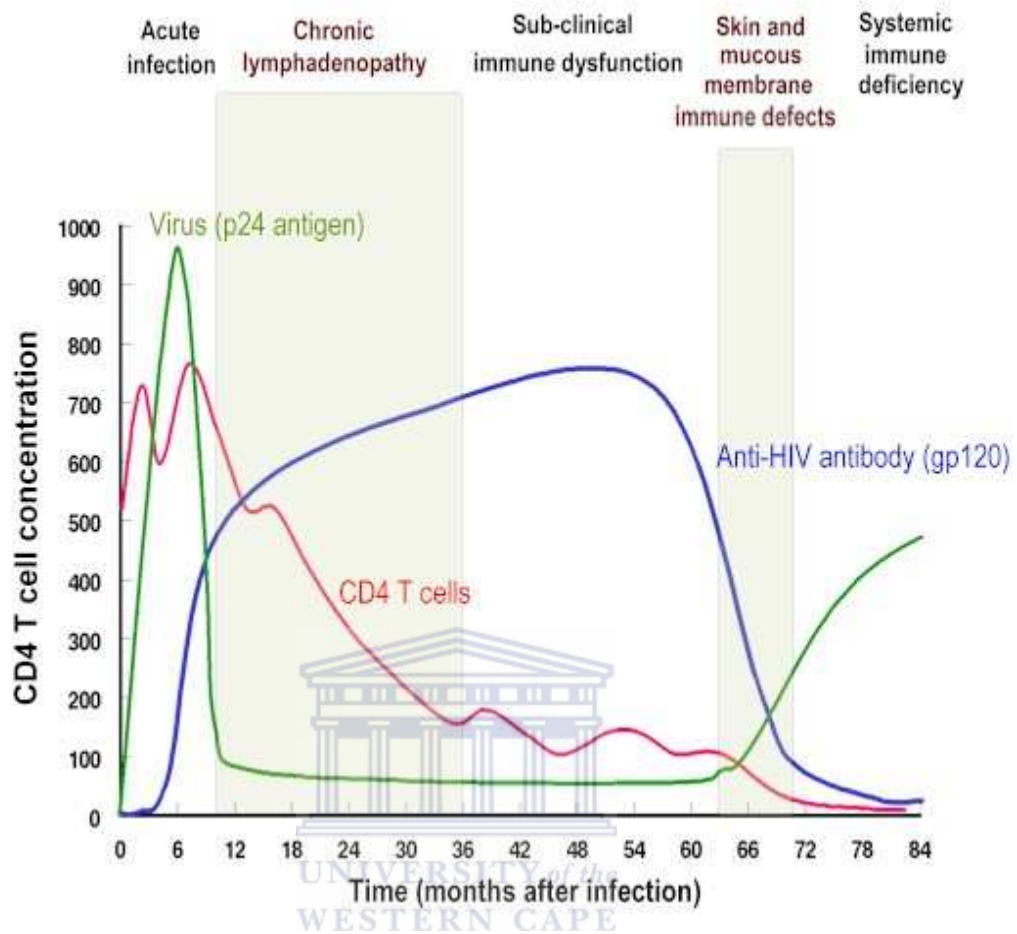
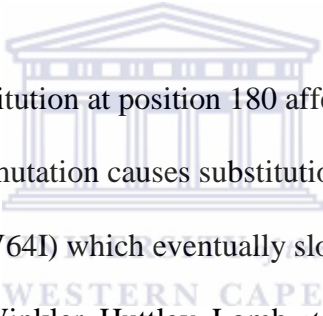


Figure 4: Natural history and progression pattern of HIV infection to AIDS in the absence of treatment (Figure taken from Fauci, 1998).

2.4 HIV and Host genetic factors

Host genetic factors, including chemokine receptors and chemokine genotypes, have been identified as having an impact on both HIV-1 infection and disease progression to death (Michael, 1999). These findings prompted more research work that discovered two sets of factors (virus related and host immunologic factors) that have profound effect on HIV-1 transmission and disease progression. The relationships between the two sets of factors have

provided valuable tools in monitoring ARV therapy (Kumud Barroga, Hughes, Chen Raskino, McKinney, 2003). Polymorphisms within coding and regulatory regions of chemokine receptors and co-receptors have an impact on HIV-1 related pathogenesis. The presence of CCR5- Δ 32, deletion of 32 base pairs from the coding region of CCR5 gene, leads to the encoding of a distorted CCR5 protein. This has been reported to provide partial protection against HIV-1 infection in people who are homozygous for mutant genotypes (Dean, Carrington, Winkler, Huttley, Smith, Allikmets, 1996; Liu, Paxton, Choe, Ceradini, Martin, Horik, 1996). Heterozygotes for a Δ 32 deletion (CCR5-wt/ Δ 32) are not protected against HIV-1 infection but manifest slow progression to HIV-1 and AIDS end points (Shearer, Kalish & Zimmerman, 1998; Kostrikis *et al.*, 1999).



On the other hand, A *G to A* substitution at position 180 affects the gene that codes for CCR2, a minor HIV-1 co-receptor. This mutation causes substitution of Isoleucine to Valine at position 64 (designated as CCR2V64I) which eventually slows down disease progression in adults (Smith, Dean, Carrington, Winkler, Huttley, Lomb *et al.*, 1997; Kostrikis *et al.*, 1999). Similarly mutations in the 153' untranslated region of the gene that codes for SDF-1 β (pre-B growth stimulating factor) lowers progression of HIV-1 infection to AIDS because SDF-1 is a natural ligand for CXCR4 which acts as a co-receptor for HIV-1 (Winkler, Modi, Smith, Nelson, Wu & Carrington, 1998; Yuan, Peterson, Wang, Goodsaid & Waters, 2000). Thus generally chemokines co-receptors seem to have some varied influence on the transmission of the AIDS causing virus.

2.5 CCR5 Δ 32 and CCR2 V64I

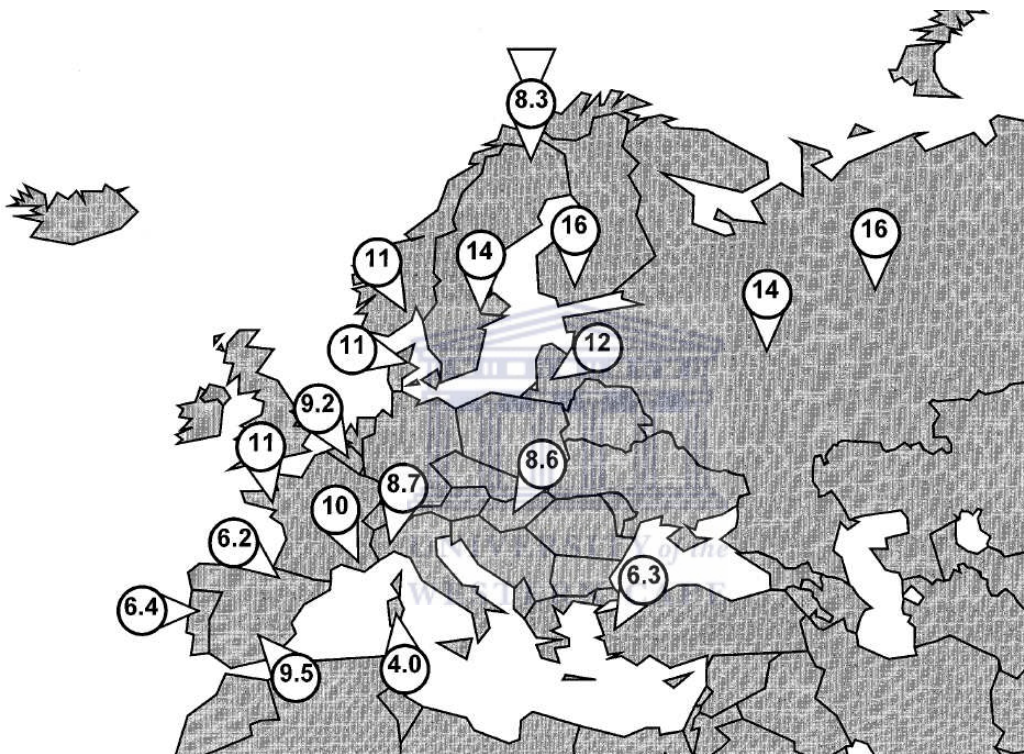
2.5.1 Trends of CCR5 Δ 32 and CCR2 V64I in populations

The global distribution of chemokine co-receptors genes among different populations has been heterogeneous, just like the distribution of the HIV infection. This scenario has led to the suggestion that there might be some underlying factors influencing susceptibility to the virus in some populations compared to others (Martinsoni *et al.*, 1997), since genetic mutation in host genes has been reported to show some strong geographical traits (Novembre, Galvani, Slatkin, 2005; Schliekelman, Garner, Slatkin, 2001).

A general North to South downhill gradient in CCR5- Δ 32 has been observed as shown in Figure 5, with only the Lapps (Saamis) constituting a notable exception. The highest frequency has been found in Northern and North Eastern Europe (16% in Finnish and Mordvinian populations). The Swedish Saamis have been reported as having a significantly lower frequency (8.3%) of CCR5 - Δ 32 gene than Swedes. The highest of the frequencies has been observed in northern Europe and the lowest in Greece (Libert *et al.*, 1998). This data confirm the high frequency of CCR5- Δ 32 among northern European Caucasians, a gene frequency cline across Europe and Asia reflecting recent population admixture, and virtual absence of CCR5- Δ 32 among native Africans, East Asians, and American Indians (Stephens *et al.*, 1998). Based on the demographic distribution, it is believed that the mutation arose in northern Europe in response to selective pressures due factors such as infection epidemics (Libert *et al.*, 1998).

Previous studies have suggested that the CCR5- Δ 32 gene was absent among Africans, American-Indians and East Asians. Subsequent research has largely sustained these geographical patterns, although geneticists have observed that the allele was not wholly

absent from non-Eurasian populations, but is also detected in people of African descent (Novembre *et al.*, 2005; Schiekelman *et al.*, 2001). In Africa, a South African study conducted by Williamson and colleagues (2000) indicates that the allelic frequency of CCR5-Δ32 mutation was 0.1% in the black South African population, suggesting that the 32 base pair gene mutation could be drifting from the north to the south due to population admixture.



Source: Libert *et al.*, 1998.

Figure 5: Frequency of the CCR5-Δ 32 allele in European populations. Schematic map of Europe indicating the frequency (in percentage) of the CCR5 mutant allele CCR5-Δ32 in the various populations.

On the other hand the frequency of CCR2V64I allele has been observed to be higher than that of CCR5-Δ32 with a prevalence rate ranging from 10% to 25% in both black and white persons and in all other ethnic groups studied (Christine *et al.*, 2001). Studies investigating

the distribution of the CCR2V64I mutation and allelic frequencies in different populations and ethnic groups worldwide (Martinsoni *et al.*, 2000; Struyf , Thoelen, Charlier, Keyaerts , Van der Donck, Wu, 2000; Mangano, Kopka, Batalla, Bologna, Sen & Barbouche, 1998; Iyer, Kim, Bando, Lu, Gregg & Grody, 2001) indicate that this mutation is most common in Asian populations (0.250), least common in Europeans (0.098), and of intermediate frequency (0.151) in African populations (Smith *et al.*, 1997). In Africa, studies carried out suggest that CCR2V64I vary among ethnic groups with a frequency of 13% particularly in South Africans, while another study showed a 23% frequency in Kenyans (Martinson *et al.*, 1997; Smith *et al.*, 1997). Further to that, Anzala and co-workers (2000) observed that the presence of the mutation helped to delay HIV-1 progression to AIDS among 21% to 46% of slow progressors in their study among infected commercial sex workers in Nairobi, Kenya.

2.5.2 Chemokine coreceptor Polymorphism and HIV-1 Infection

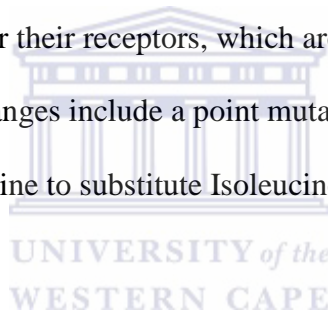
2.5.2.1 CCR5- Δ 32 polymorphism and HIV infection

The macrophage-tropic HIV-1 uses the CCR5 to infect macrophages. The chemokine co-receptors 5 (CCR5) gene has been identified as the main co-receptors that, in conjunction with CD4, aids the viral fusion and infection of human host cells by macro-phagic-tropic human immunodeficiency virus type 1 (HIV-1) strains (Deng, Liu, Ellmeier, Choe, Unutmaz, Burhart & Di Marzio, 1996; Dragic, Litwin, Allaway, Martin, Huang & Nagashima, 1996). Over the past few years, a number of mutations in CCR5 have been identified as natural genetic polymorphism able to influence the probability of acquiring HIV-1 infection or to affect the rate of disease progression, in infected adults (Kostrikis *et al.*, 1999) or infants (Misrahi *et al.*, 1998), though the allelic composition of the gene plays a crucial role in determining the outcome of exposure. Numerous studies now support the hypothesis that the homozygosis for a 32 base pair deletion (Δ 32) within the CCR5 gene is associated with complete resistance to HIV-1 infection (Liu *et al.*, 1996). Liu and co-workers, (1996)

observed that individuals homozygous to CCR5-Δ32 gene remained uninfected with HIV-1 despite multiple exposures to the virus. Further to that, Michael and collaborators (2001) demonstrated that CCR5-Δ32 homozygous gene was associated with protection against HIV infection in a cross-sectional and longitudinal analysis of a cohort of 2996 of initially HIV sero-negative individuals comprising of homosexual men, male and female injection drug users (UDIs) and heterosexual risky women in North America.

2.5.2.2 CCR2V64I polymorphism and HIV infection

During the last five years, several communications have been published about the discovery of hereditary alterations of human genome determining resistance to HIV-1 and/or the rate of disease progression. The genome changes have been associated with the genes encoding chemokines or their receptors, which are secondary HIV-1 attachment sites to the immune cells. The changes include a point mutation at position 64 of the CCR2 receptor gene (causing Valine to substitute Isoleucine on the receptor protein).



The protective effect of CCR2 V64I against HIV infection has been controversial. A number of studies have observed that adults who possess the CCR2V64I allele (isoleucine substitution for valine at position 64) are not protected against HIV-1 infection, but progress less rapidly to disease once infected (Christine *et al.*, 2001). Others suggest that CCR2V64I interferes with CCR5 co-receptors expression; however this hypothesis was rejected by a recent *in vitro* study showing that the CCR2V64I allele does not influence CCR5 transcription or mRNA levels (Matte & Roger, 2001). Contrary to that, a study of infected commercial sex workers in Nairobi, Kenya, suggests that the presence of the mutation helped to delay progression of HIV-1 infection to AIDS in 21% to 46% of slow progressors (Anzala, Ball, Rostron, O'Brien, Plummer, & Rowland-Jones, 1998).

2.5.3 Chemokine receptors in HIV-1 therapeutic development

Targeting of viral co-receptors to block HIV entry has been a promising approach to prevent and treat HIV-1 infection. The human immunodeficiency virus 1 (HIV-1) enters CD4 + cells by fusion at the plasma membrane after interactions with CD4 and co-receptor molecules (Berger, 1997). The first co-receptor to be identified was CXC chemokine receptor CXCR4 (Feng, 1996), closely followed by the CC chemokine receptor CCR5 (Alkhatib, Combadiere, Broder, Feng, Kennedy, Murphy, 1996). These two proteins are generally considered to be the most important co-receptors used by HIV-1 strains of the T-tropic and M-tropic phenotypes, respectively (Berger, 1997).

Nature has provided a model for blocking CCR5, that is, people who express a deletion mutation of CCR5 (CCR5-Δ32) on their cell surfaces. The protective property of CCR5-Δ32 against HIV infection has prompted pharmaceutical companies to develop a CCR5 antagonist for clinical use (Clotet, 2007). One such CCR5 antagonist that has been developed recently is Maraviroc.

Maraviroc is a CCR5 antagonist that selectively binds to the human chemokine receptor CCR5, present on the cell membrane, preventing the interaction of HIV-1 gp 120 and CCR5 necessary for CCR5-tropic HIV-1 to enter cells. The drug has shown potent activity against multidrug-resistance CCR5-tropic HIV-1 strains. It has antiviral activity *in vitro* against CCR5 tropic HIV-1, including 43 HIV-1 isolates from various clades and geographic regions, as well as 200 HIV-1 envelope-derived pseudoviruses. Maraviroc has no activity against CXCR4-tropic and dual tropic HIV-1 (Sayana & Khanlou, 2008). Data gathered from human drug trials with Maraviroc demonstrates that it is efficient and safe to use in combination with

other antiretroviral agents for treatment-experienced adults infected with only CCR5 tropic HIV-1, who have evidence of viral replication (Sayana & Khanlou, 2008).

Despite the availability of evidence linking some host genetic factors to protection against HIV infection, very little information is still available on the role of CCR5- Δ 32 and CCR2V64I polymorphism on HIV transmission in Africa's high endemic areas (Williamson *et al* 2000), like Zimbabwe. Yet the current HIV situation suggest that the very future of Africa is threatened (Kaul, Makadzange & Rowland-Jones, 2000) and perinatal HIV-1 infection constitutes a significant global health problem and the prevention of transmission has to be a high public health priority. Therefore, knowing the range of co-receptors that could be used by HIV-1 in *in vivo* is important for the development of antiviral drugs aimed at inhibiting HIV-1 entry.



CHAPTER 3: METHODOLOGY

3.1 Aim

The aim of the study was to determine and compare the prevalence of homozygous CCR2V641 and homozygous CCR5- Δ 32 mutations in a HIV positive and HIV negative population of pregnant women from Harare, in Zimbabwe.

3.1.1 Objectives

The objectives of the study were:

- To determine and compare the prevalence of homozygous CCR5- Δ 32 mutation in a cohort of HIV positive and HIV negative population of pregnant women from Harare in Zimbabwe.
- To determine and compare the prevalence of homozygous CCR2V641 mutation in a cohort of HIV positive and HIV negative population of pregnant women from Harare in Zimbabwe.
- To assess if there is any association between homozygous CCR2V641 and homozygous CCR5 - Δ 32 mutations with HIV infection in a cohort of HIV positive and HIV negative population of pregnant women from Harare in Zimbabwe.
- To assess if sexual transmitted infections had any influence on the outcome of the current study.

3.2 Study design

This was a cross-sectional study of homozygous CCR2V641 and homozygous CCR5 - Δ 32 mutations in which measurement of exposure (chemokine co-receptor status) and effect (HIV-status) were made at the same time. The current study had to adopt this design because

it is relatively easy and economical to conduct and is useful in investigating exposures that are fixed characteristics like the chemokine co-receptor genes.

3.3 Definition of terms

CCR2V64I- A substitution of valine for isoleucine in the CCR2 receptor gene, resulting in normal levels but altered first transmembrane region of the CCR2 receptor, which has been associated with delayed progression of HIV-related illness.

CCR5Δ-32- A 32 base deletion in the CCR5 gene that results in a shortened and non-functional protein. Homozygosity for CCR5-Δ32 is associated with decreased susceptibility to HIV, and heterozygosity is associated with delayed progression of HIV-related illness.

CCR5-A chemokine receptor present on macrophages, monocytes, and some T cells, particularly memory T cells. Along with CD4, CCR5 acts as a co-receptor for M-tropic HIV.

CXCR4- Chemokine receptor located on T cells, particularly naive T cells, which, along with CD4, acts a co-receptor for T-tropic HIV.

Chemokine co-receptor –These are molecules that are found on CD4 -T cell which in conjunction with CD4 molecules cell facilitate host cell entry by the HIV.

Polymorphism- Different forms of a gene existing in different forms caused by gene mutation or deletions.

Macrophage-tropic (M-tropic) – HIV variants with a non-syntium-inducing phenotype that infect monocyte –derived macrophages *in vitro* but not established CD4⁺ T-Cell lines. Also called R5 *variants* because they use the CCR5 co-receptor for entry, they tend to be the predominant variant during early infection.

T-cell-tropic HIV – HIV variants with a syncytium-inducing phenotype that infects established CD4 T-cell lines. Also called X4 variants because they use the CXCR4 co-receptor for entry, they tend to emerge during a later stage of HIV infection.

Host genetic factors- These are inherited genetic materials that lead to the encoding of proteins or amino acids that confer protection or pre-dispose one to HIV infection.

Wild type gene- A gene without mutation either of a pair (or series) of alternative forms of a gene

Allele- An allele is one of a pair of alternative forms on the DNA sequence of a particular gene . An allele can be RR (dominant), Rr (dominant), and dd (recessive). Alleles can be homozygous (same)-RR and rr or heterozygous (different)-Rr.

Validity- Validity determines whether the research truly measures that which it was intended to measure or how truthful the research results are.

Reliability- Is the extent to which the results of a study are consistent over time and is an accurate representation of the total population under study. Results of a reliable study should be reproduced under a similar condition and methodology.

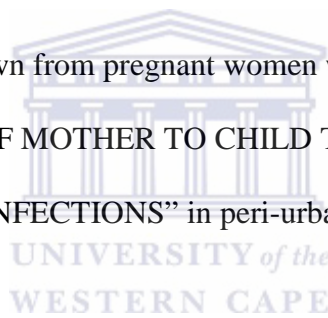
Main study- An ongoing study from which the current study drew its samples from.

STIs – These are sexual transmitted infections (genital, warts, genital ulcers, HVS-2, *Trichomonas vaginalis* and syphilis)

3.4 Study population

This was a baseline survey of sera samples collected from pregnant women in three clinics namely Epworth, St Marys' and Seke North in Harare. The 76 HIV positive sera samples were randomly drawn from 456 HIV infected pregnant women, while 166 HIV negative sera samples were randomly selected from 594 HIV uninfected pregnant women. The selection of samples was done using an existing sample register and 166 samples were supposed to be selected from each group. The number had been inflated in order to cater for attrition due to method failure. However, positive samples ended up being less than the negative samples because most of the samples were insufficient to conduct the assays.

The serum samples had been drawn from pregnant women who had participated in the main study entitled "PREVENTION OF MOTHER TO CHILD TRANSMISSION OF SEXUALLY TRANSMITTED INFECTIONS" in peri-urban Harare between 2002 and 2004.



3.4.1 Inclusion criteria

The study population from where the sera samples were drawn from were pregnant women of any race residing in Zimbabwe with a minimal age of 18 years using Epworth, St Mary's and Seke North clinics for ANC. These clinics have been selected as sites for the main study because they have been reported to have a higher prevalence of HIV infection in Harare.

3.4.2 Exclusion criteria

The main study excluded women who were not pregnant. As a result our sera samples were all from pregnant women.

3.5 Sampling

3.5.1 Sample size calculation

Sample size for this sub-study was determined using sample size calculation for single proportion. The formula for the calculation is as given below.

$$\text{Sample size} = z_{\alpha}^2 / \Delta^2 \times p(1-p)$$

Where $p = 25\%$ and is the assumed prevalence of CCR2V641 gene polymorphism in Zimbabwe. This was based on the prevalence rates from the study by Matte *et al.*, (2001) who observed that the prevalence of CCR2V641 ranged from 10-25% in both black and whites as the exact prevalence of the gene is not known in Zimbabwe.

Δ = The precision, in this case was set at 0.05

Where $z_{\alpha} = 1.96$

Sample size = 288



When the sample size was calculated using the lower limit prevalence rate of $p=10\%$ based again on Matte *et al.*, (2001) study, a sample size of 138 was produced. It follows that the possible range of the acceptable sample size lied between 138-288 samples.

3.5.2 Controlling for sampling bias

242 samples were randomly selected from 1050 serum samples for this study. 76 HIV positive samples were randomly selected from 456 HIV positive serum samples, while 166 HIV negative samples were randomly selected from 594 HIV negative serum samples, all available from the main study. All serum samples were collected from pregnant women who had visited the clinic to register as well as for routine antenatal clinic visits and later on volunteered to participate in the main study. It is therefore assumed that these 242 samples

represented the host genes to HIV infection of the 1050 samples from pregnant African women in peri-urban Harare.

3.6 DATA COLLECTION

3.6.1 Demographic data

General demographic data consisting of age, race, and sexual transmitted disease status for mothers with randomly selected samples were drawn from the existing main study register.

3.6.2 Blood samples

Blood samples in the main study were collected as follows:

-5ml of blood was drawn from the venepuncture into an anticoagulant free sample tube for sera separation.



3.6.3 Laboratory methods

3.6.3.1 Sample collection and processing

The serum samples that were used in this study were part of the 1050 samples collected from the parent study entitled “PREVENTION OF MOTHER TO CHILD TRANSMISSION OF SEXUALLY TRANSMITTED INFECTIONS”. In brief 5 ml of whole-blood was collected in an anticoagulant free VACUTAINER tubes for each person. The clotted whole-blood samples were centrifuged for 20 minutes at 1,200 g in a swinging bucket rotor (RT 6000B; Sorvall) centrifuge. Sera was then aliquoted into cryovials (Nalgene, Nunc International, Roskilde, Denmark) and stored at -70°C in a deep freezer. After extraction the samples were then shipped on dry ice to the institute of Microbiology in Oslo in Norway for CCR5- $\Delta 32$ and CCR2V641 polymorphism determination.

3.6.3.2 HIV testing

HIV status of the samples was drawn from the main laboratory result register. These samples had been tested as follows:

- Serial HIV-1/2 algorithm antibody tests were done using Determine (Abbot Diagnostics, Illinois, USA) and Ora-Quick (Abbot Diagnostics, Illinois, USA) rapid kits on mother sera samples in the main study.

3.6.3.3 STIs screening

Antibodies to HSV-2 at baseline were identified using the HerSelect 2 ELISA IgG (Focus Diagnostics) and the results were interpreted according to the manufacturer's specifications. Screening of syphilis was done using a non-specific rapid plasma reagin (RPR) and all positive were confirmed using Triponema Pallidum Hemagglutination Assay (TPHA), both manufactured by Randox Laboratories. A wet mount prepared from freshly collected vaginal fluid was mixed with normal saline and examined using a compound microscope for the presence of *Trichomonas vaginalis* and yeast cells.

3.6.3.4 CCR5-Δ32 and CCR2V64I polymorphism determination using PCR

DNA was extracted from serum samples following the guanidine isothiocyanate acidified silica procedure of Boom, Sol, Salisman, Jansen, Wertheim-van Dillen and van der Noorda (1990) (see Appendix 7 for procedure).

3.6.3.5 PCR controls

Interpretation of assay results was based on positive controls that were simultaneously tested together with the samples. In the current study, plasmids containing the gene of interest were used as controls for both the CCR5-Δ32 and CCR2V64I PCR assays. The plasmids were obtained from Eurofins MWG Operon in Germany (see appendices 10, 11, 12 & 13)

The CCR5 wild type plasmids had a 200 base pair (bp) insert of the CCR5-Δ32 gene (see appendix 10). The CCR5-Δ32 gene had a 32 base pair deletion, resulting in a 168 bp product (see appendix 11). The CCR2 wild type and CCR2V64I plasmid contained a 380 bp insert (see Appendix 12 & 13).



Figure 6: A cabinet where the sample extraction was done from. Working under the cabinet was necessary to maintain aseptic conditions in order to avoid contamination of samples with foreign- DNA.

3.6.3.6 Agarose gel electrophoresis

To assess whether the DNA was successfully extracted from the samples before testing for chemokine co-receptors, 10 % of the samples were amplified and PCR products were then loaded on 2 % agarose gel and electrophoresed. This was done by mixing 10 μ L of DNA elute (about 5 μ g) with 1 μ L of 1X Loading Buffer (30% Glycerol, 0.25% Bromophenol Blue, 0.25% Xylene cyanol) and loaded onto 2.0 % (w/v) agarose gel in 1X Tris-Borate EDTA (TBE) diluted appropriately from the TBE stock solution, 0.054g Tris-Base, pH 8, 27.5g boric acid, 20 ml of 0.5 M EDTA, pH 8.0) (see Appendix 7). The gel was electrophoresed for a run time of 1 hour at a constant power of 100 V using Bio-Rad PowerPac 1000 (Bio-Rad, Ivry-sur-Seine, France).

The genotyping and amplification of CCR5- Δ 32 and CCR2V641 polymorphism were performed using a simple and fast automated method based on real-time PCR on an Applied Biosystems Step One Plus Real-Time PCR system (Figure 7) (Applied Biosystems, Foster City, California, USA) and Light CyclerTM technology (Roche Diagnostic, Rahway, NJ, USA) respectively. The method had two steps: (1) real-time PCR amplification and (2) melting curve determination.

3.6.3.7 Real time PCR amplification

During this process, amplification and fluorescent detection steps were done in the same tube and data recorded online. The PCR instrument then measured the accumulation of PCR products during amplification with the fluorescent dyes.

3.6.3.8 Principle of SYBR green technology and melting curve

SYBR green binds to the double-stranded DNA, and upon excitation emits light which can be measured at a certain wavelength. Thus as the PCR product accumulates, fluorescence increases. However, when SYBR green double-stranded complex is heated, the double

stranded DNA dissociate at different rates, since the energy required to break the base-base hydrogen bonding between two strands of DNA is dependent on their length, GC and their complementarity. The dissociation of the DNA during heating is measured by the large reduction in fluorescence that results in SYBR enabled product on Applied Biosystem StepOne™ Real time -PCR instrument. Genotypes homozygous for the mutant allele have a low melting point than do genotypes wild type for the mutant allele.

3.6.3.9 Melting curve determination

During the melting curve analysis with SYBR Green, products were characterised, in order to determine whether they had a deletion or not. PCR products were characterised by melting curve analysis because each double-stranded DNA molecule had a characteristic melting temperature (T_m), at which 50% of the DNA is double- stranded and 50% is melted, i.e, single-stranded. During a melting curve run, the reaction mixture was slowly heated to 95⁰C, which caused dsDNA to melt. A sharp decrease in SYBR Green fluorescence occurs when the temperature reach the T_m of a PCR product present in the reaction. The Applied Biosystems Step One™ Plus Real-time PCR machine continuously monitor fluorescence during temperature transitions. The computer software displayed these data as a melting curve graph (fluorescence [F] vs. Temperature [T] (Figure 8).



Figure 7: A Medical Research Technologist testing the genomic samples for CCR5- Δ 32 mutation on Applied Biosystem StepOne™ Real-time PCR instrument at Oslo University in Norway.

In order to determine the possible appearance of CCR5- Δ 32 alleles, real time PCR was performed on Applied Biosystems Step One Plus Real-time PCR system using a double strand-specific dye SYBRGreen®. Primers flanking the CCR5- Δ 32 mutation were D32-F2, 5'TTCATTACACCTgCAgCTCTC3' and D32-R, 5'gACCAg CCCCAAgATgACTATC-3'. (TIB MOLBIOL Syntheselabor GmbH, Germany) (see Appendix 3 for primer specifications). Each 20 μ l reaction mix contained 5 μ l of genomic DNA; 10 μ l of 2x SYBR

Green mix (POWER SYBR[®] GREEN PCR MASTER MIX); and 3 μ l (10 pmol) of each primer (D32F2 and D32 R). Forty cycles of amplification were performed in three different stages consisting of various steps in an Applied Biosystems Step One Plus Real-time PCR machine as describe below:

Stage 1: Activation of enzyme

Step 1: 95⁰C for 10:0 minutes

Stage 2: PCR x 40

Step1: 95⁰C for 0:15s

Step 2: 60⁰C for 1:00 minutes (min)

Stage 3: Melting curve

Step 1: 95⁰C for 0:15s

Step 2: 60⁰C for 1:00 min, then 0.3⁰C/slope up to 95⁰C

Step 3: 95⁰C for 0:15s

Fluorescence was measured at 520nm of the fluorometer. Amplicons containing the 32 –bp deletion were supposed to have a melting temperature (T_m) which was approximately 2⁰C lower than that of the 83 bp (Figure 8) (Ruiz, 2001). Genotypes homozygous for the mutant allele had a low melting point than do geneotypes wild type for the mutant allele (72.2 versus 74.28⁰C).

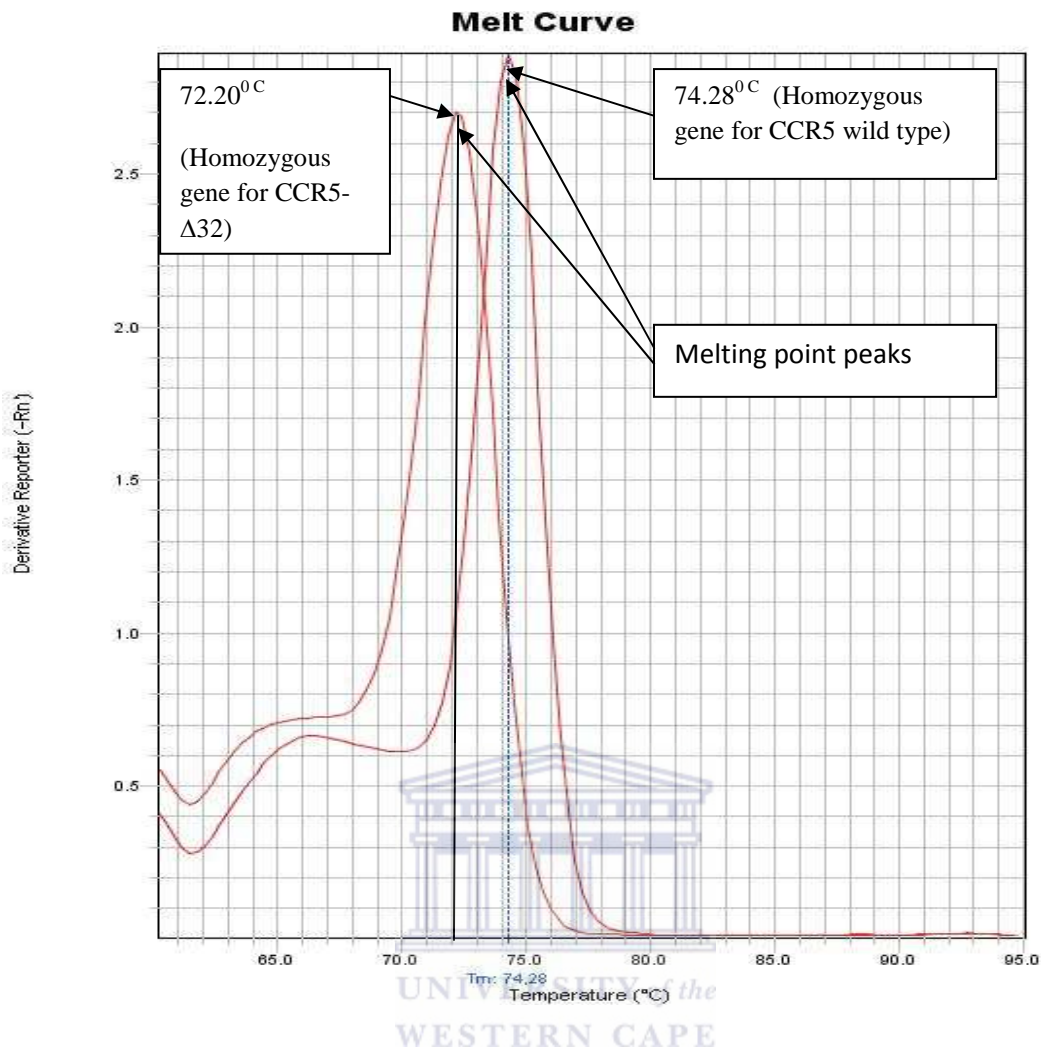


Figure 8: Analysis of the fluorescent measured during melting curve determination of CCR5 in Applied Biosystems Step One™ Plus Real-time machine. Genotypes homozygous for the mutant allele had a low melting point than do genotypes wild type for the mutant allele (72.2 versus 74.28°C).

For CCR2V64I genotyping, real-time PCR was performed in the LightCycle™ system.

The determination of CCR2V64I in a LightCycle™ system was based on Fluorescence Resonance Energy Transfer (FRET).

3.6.3.10 FRET Principle

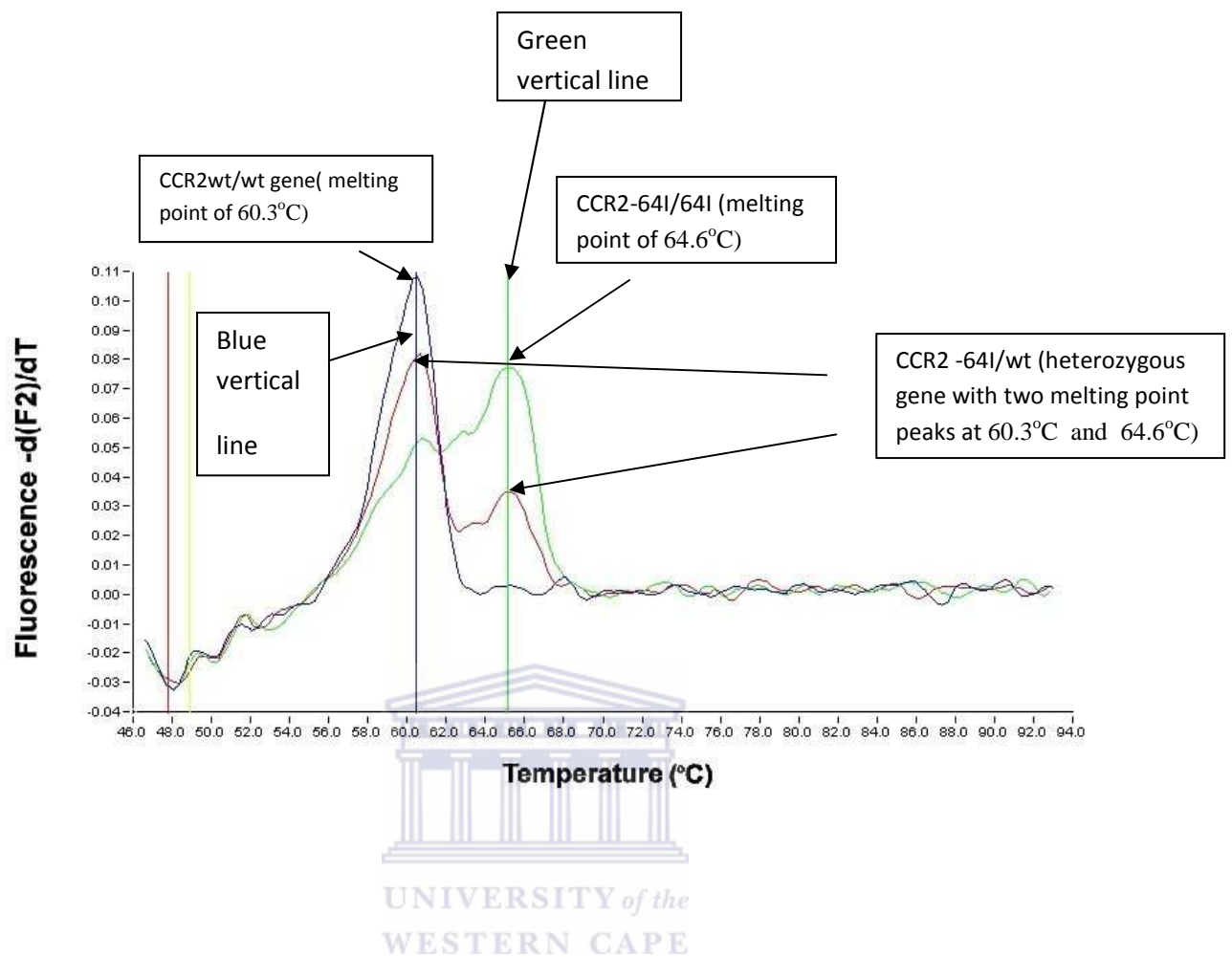
FRET analysis is based on the transfer of energy from one fluorescent molecule (e.g. fluorescein) to another adjacent fluorescent molecule (e.g. LightCycler rTM system Red 640). In FRET, fluorescein is excited by blue light, leading to the emission of fluorescent light with a wavelength of 530 nm and transfers of this energy to LightCyclerTM 640 molecule (which is not directly affected by the blue light). The LightCyclerTM 640 molecule is in turn excited and emits light which is measured at 640 nm.

Two different oligonucleotides (anchor and sensor) hybridize, head to tail, to adjacent regions of the target DNA, making possible the fluorescence resonance energy transfer (FRET) reaction. Due to the sequence specificity, artificial by-products such as primer-dimers or PCR by products are not detected.

Flanking primers and internal probes were designed according to the sequences of interest (CCR2-F, ATgCTgTCCACATCTCgTTC-3' and CCR2-R, 5'-CCCAAAGACCCACTCATTTg-3'; CCR2Sensor-mt, 5'AACATgCTggTCATCCTCATCTTAATAA-FL and CCR2 Anchor, LC640-CTgCAAAAAGCTgAAgTgCTTgACTg-PH) (TIB MOLBIOL Syntheselabor GmbH, Germany) (see Appendices 4 & 5 for primer and probe specifications). Each 20 µl PCR contained 2 µl of genomic DNA, 2 µl of Hyb Prob Enzyme-Master mix; 3 mM MgCl₂ ; 2 pmol of each Sensor and Anchor probes; and 10 pmol of each primer (CCR2-F and CCR2-R). Denaturations were conducted for 2 minutes and were followed by 45 cycles of amplification (95 °C for 0s, 64 °C for 15s, 58 °C for 25s and 72 °C for 25s). Amplification was then followed by determination of the melting curve (95 °C for 20s, 64 °C for 15 s, and 58 °C for 25s and from 45 °C to 95 °C, with a 0.2 °C/s slope), fluorescence was measured on

channels F2 (640 nm)/ F1 (520) of the fluorometer. During the temperature curve determination the melting point of the sensor was measured and depended on the presents/absence of mismatch along sequence (mutant vs wild-type allele). Amplicons containing the wild-type sequence was supposed to have an approximate melting point of 4⁰C lower than amplicons with the polymorphism as shown below in Figure 9.





Blue vertical line- Melting point for CCR2wt/wt (wild type gene)

Green vertical line- Melting point for CCR2-64I/64I (homozygous mutant gene)

Figure 9: CCR2V64I analysis by Real time PCR. Analysis of the fluorescent measured during melting curve determination in the LightCycler system. Genotypes homozygous for the wild- type (wt) allele had a lower melting point than do genotype homozygous for mutant alleles (CCR2V64I/64I) (60.3°C vs 64.6°C)

3.7 Reliability

Before the inception of the study the conductor and author of this study underwent training in the conducting of PCR technique in Norway for a month in 2009. A pilot study to test the data collection tools was done before the initiation of the laboratory tests. Constant

supervision of the conductor's laboratory work was done by an expert molecular biologist from Oslo University to ensure that data of highest quality was collected.

Reliability was also ensured through the inclusion of positive and negative controls during testing in every test batch. These controls guided us on whether to accept or reject the assay results. Assays that were in disagreement with the controls were rejected and repeated.

3.8 Validity

The current study intended to investigate whether CCR5-Δ32 and CCR2V641 chemokine co-receptors were a risk factor for HIV infection. We adopted a number of factors to ensure validity. Firstly we used the real-time PCR technique to determine the chemokine co-receptor genes in sera samples instead of the conventional PCR end-point method. This technique has a number of advantages over other PCR methods, like the end-point method e.g the technique is much faster. In addition to that real-time PCR produces reliable results, it is far more sensitive than ethidium bromide staining detection. Finally real-time PCR minimises the chance of contamination because both amplification and detection are conducted in a single closed tube (Leindios, Tyagi, Mhlanga, Ho, Kramer, 1998).

3.9 Accounting for bias and confounding factors

3.9.1 Information bias

Although a questionnaire was administered to the participants to capture information on whether participants were suffering or had ever suffered from sexual transmitted diseases, the study could not rely on that information only, and thus samples were further examined by additional laboratory tests.

3.9.2 Confounding

To account for possible influence of confounders like STIs (sexual transmitted infections), multivariate regression analysis was used in the analysis of data. However, there may be other confounders like the nutritional status, number of sexual partners among others that could not be accounted for in this study.

3.10 DATA ANALYSIS

Data was entered and analysed using SPSS program. Baseline descriptions of demographic characteristics were compared between mothers that had CCR5-Δ32 and CCR2V641 chemokine co-receptors and those who did not. Chi-square (χ^2) tests and relative risks for CCR5-Δ32 and CCR2V641 chemokine co-receptors were calculated. Thereafter, factors that had a *p*-value of less than 0.25 in the univariate analysis were included in multivariate regression analysis to control for their confounding effect.

3.11 Ethical considerations

This study was approved by the Medical Research Council of Zimbabwe (MRCZ) (Ref MRCZ/A1407). Host genetic factors to HIV study is part of the HIV-1 Genetic diversity study and both are sub-studies of the main study entitled “PREVENTION OF MOTHER TO CHILD TRANSMISSION OF SEXUALLY TRANSMITTED INFECTIONS”.

Participation in the main study and the Host gene study was absolutely voluntary. Prior to enrolment, the entire consent form (Appendices 1 & 2) was read to the participants either in Shona or English languages as the case may be. Refusing to have any of one's sample stored other than being used to complete the main study did not prejudice the participants' involvement in any other future studies by us.

CHAPTER 4: RESULTS

4.1 Demographic data

Chemokine co-receptors results of CCR5-Δ32 and CCR2V64I genes were available from 242 Black African pregnant women (mean age 25.0 and 95% CI =24. 3-25.6 years). HIV infected mothers were significantly older (mean age 26.7, 95% CI = 25.6-27.7 versus mean age 24.1, 95% CI =23.3-24.9, $p<0.001$) than HIV negative.

4.2 Confirmation of DNA extraction from serum samples

90% of the serum samples that were amplified and PCR products gel electrophoresed produced bands similar to those shown in Figure 10 below.



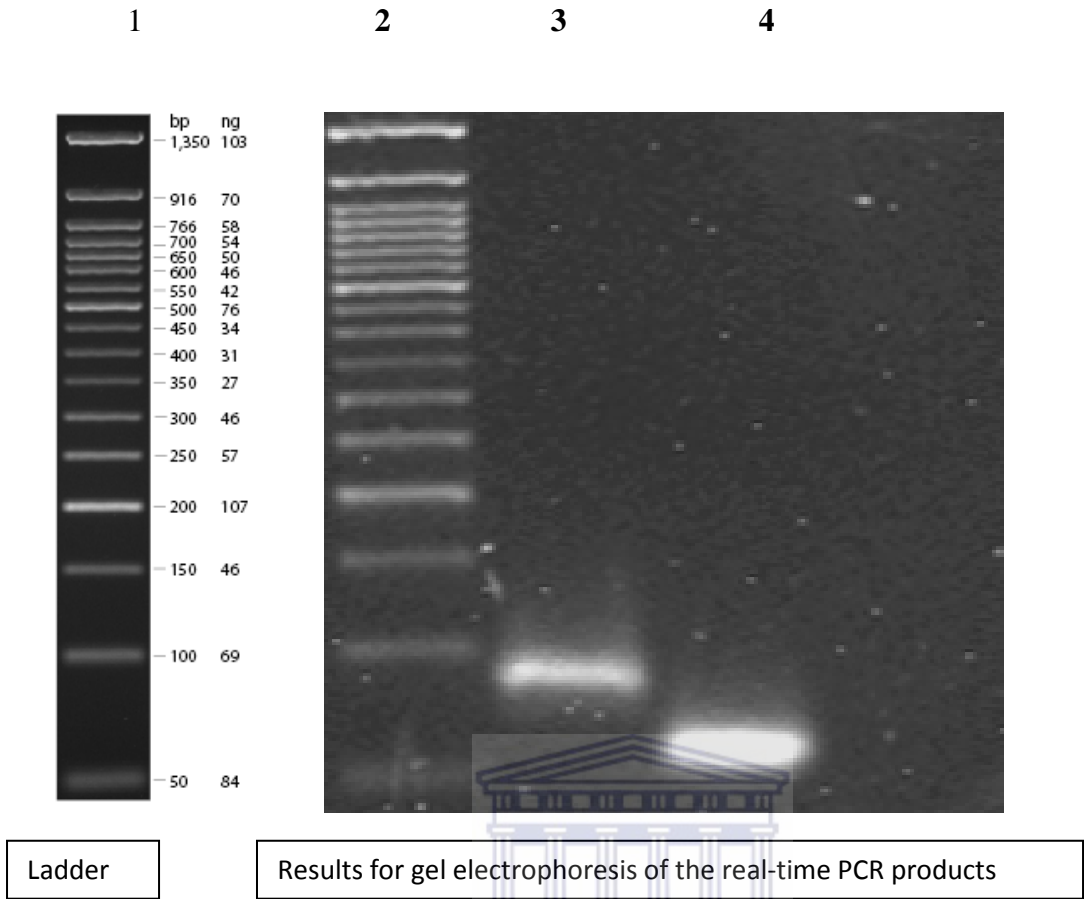


Figure 10: Electrophoresis in a 2% agarose gel of the amplicons resulting from real-time PCR. Lane 1 and 2 represents marker V (Roche), lanes 3 and 4 show DNA bands for samples from participants.

4.3 Prevalence of chemokines co-receptors and STIs

None of the women had a CCR5-Δ32 homozygous gene (Table 1). 76 of the 242 sera samples were drawn from HIV positive mothers, while the remainder was collected from HIV negative mothers. The overall prevalence of CCR2V64I homozygous gene was 24.4 % (n=59, 95% CI =18.93-29.83). Of the 59 individuals with CCR2V64I homozygous gene, 49.15% (n=29) were HIV positive while the remainder was HIV negative. Results for heterozygous genes for both CCR5-Δ32 and CCR2V64 genes could not be analysed because we had no controls for heterozygous genes to authenticate our laboratory results.

Table 1: Genotype and allelic frequencies of CCR2 and CCR5 genes within pregnant women.

Variable	wt/wt ^a	wt/mt ^b	mt/mt ^c	Mutated allele frequency (%)
CCR2	183	ND	59	24.38%
CCR5	242	ND	0	0%

^aWild type homozygous (CCR5wt/ wt or CCR2wt/wt)

^bHeterozygous (CCR5Δ32/ wt or CCR2V64I/64V)

^cMutant type homozygous (CCR5-Δ32/ Δ-32 or CCR2V64I/64I)

ND- Not determined



On the other hand STIs like HSV-2 had the highest prevalence (51.5 %) (95% CI= 44.55-58.39), while syphilis had the lowest prevalence (0.5 %, 95% CI=0.5-1.5) as shown in table 2.

Table 2: Prevalence of STIs among the study population.

Variable	Number (n)	Prevalence (%)	95% Confidence Interval (95% CI)		
			Lower limit	-	Upper limit
HSV-2	204	51.5	44.55	-	58.39
Candida albicans	218	34.86	28.49	-	41.24
Bacterial vaginosis	170	24.12	17.62	-	30.61
Trichomonas vaginalis	218	11.47	7.20	-	15.73
Genital warts	206	5.8	2.6	-	9.1
Genital ulcers	201	2.5	0.3	-	4.7
Syphilis	193	0.5	0.5	-	1.5



4.4 Factors associated with HIV infection

In univariate analysis, mothers with CCR2V64I gene were two times more likely to contract HIV infection (RR=1.913, CI= 1.338-2.737, p=0.001) compared to those without the gene.

All STIs (genital ulcers, HSV-2, *Trichomonas vaginalis* and syphilis) with the exception of genital warts were risk factors for HIV infection as illustrated in Table 3 below.

Table 3: Univariate analysis factors associated with HIV positivity among pregnant women.

Variable	HIV positive		Relative Risk (RR) unadjusted (95% CI)	p-value	
	Yes	No			
Total	76	166			
CCR2V64I	Yes	29	30	1	
	No	47	136	1.91 (1.33-2.73)	0.001
Genital ulcers*	Yes	4	1	1	
	No	52	144	3.02 (1.83-4.95)	0.008
Genital warts*	Yes	4	8	1	
	No	53	141	1.22 (0.53-2.81)	0.651
HSV-2*	Yes	52	53	1	
	No	5	94	9.81 (4.09-23.54)	<0.001
Trichomonas vaginalis*	Yes	13	12	1	
	No	41	152	2.45 (1.54-3.89)	0.001
Syphilis*	Yes	1	0	1	
	No	41	151	4.68 (3.57-6.14)	0.057

*Denominator less than 242

Further to that age was not associated with HIV infection in this study (RR=1, CI=1.00-1.10)

To account for the confounding effects of other factors like STIs, further analysis were conducted using the multivariate regression analysis (Table 4) on variables that had shown a p-value of less than 0.25 in univariate analysis. In multivariate regression analysis participants with CCR2V64I gene were still two times (RR= 2.321, 95% CI=1.376-3.916) more likely to contract HIV infection than those without the gene. Other STIs were still associated with HIV infection.

Table 4: Multivariate analysis of factors associated with HIV positivity among pregnant women using logistic regression analysis.

Variable	HIV positive		Relative Risk (RR) adjusted (95% CI)	p-value
	Yes	No		
Total	76	166		
CCR2V64I	Yes	29	30	1
	No	47	136	2.32 (1.38-3.92)
Genital ulcers*	Yes	4	1	1
	No	52	144	2.20 (1.44-3.38)
HSV-2*	Yes	52	53	1
	No	5	94	8.66 (2.66-28.21)
Trichomonas vaginalis*	Yes	13	12	1
	No	41	152	1.71 (1.06-2.76)
Syphilis*	Yes	1	0	1
	No	41	151	3.56 (1.69-7.53)

*Denominator less than 242

CHAPTER 5: DISCUSSION

5.1 Major findings of study

The major findings of this study were that homozygous CCR2V64 gene and STIs were more prevalent in HIV infected pregnant women than in those who were not infected. This study also demonstrated that homozygous CCR5-Δ32 gene was absent from these pregnant women from Harare.

5.2 Frequency of chemokines co-receptors

5.2.1 CCR2 V64I gene

The frequency of 24.38% in homozygous CCR2V64I gene in this study confirms what previous studies have reported that the gene had a frequency of 10% to 25% in all people studied (both blacks and whites) (Christine *et al.*, 2001). This study is also in agreement with studies carried out in Kenya that show a CCR2V64I gene frequency of 23% among the Kenyans (Martinson *et al.*, 1997). Similarly, Anzala and co-workers, (1998) conducted a study on infected commercial sex workers in Nairobi, Kenya, and observed that the presence of the mutation could help to explain the slow progression in 21% to 46% of the slow progressors. On the other hand, in South Africa, the frequency of CCR2V64I gene was almost two times less (13%) than in the current study (Smith *et al.*, 1997) among South Africans.

5.2.2 CCR5Δ32 gene

In contrast to CCR2V64I gene, no homozygous CCR5-Δ 32 allele was detected in any of the samples that were studied from Harare. The results augur well with other studies that have been conducted worldwide on CCR5-Δ 32 being absent in homogenous black populations, except in cases where there has been a gene flow from the Caucasian (white) to black population (Stephens *et al.*, 1998).

Elsewhere, other studies have recorded a negligible prevalence of CCR5-Δ32 gene in some African populations. A study done on African-Americans in US among people of mixed HIV infections showed a CCR5-Δ32 allele frequency of 0.017 (Dean *et al.*, 1996). While Liu and co-workers (1996) showed a prevalence of 0.2% for the CCR5 mutation in black Americans from their study in the USA.

In Africa the CCR5-Δ32 gene frequency has been less than 1%. For instance in South Africa, a study carried out by Williamson and colleagues (2000) showed the allelic frequency of the CCR5Δ32 mutation of about 0.1% in the black South African population (n=1247). In agreement with this study, a Cameroonian study (Torimino, Wolfe, Thomas, Martin, Mpoudi-Ngole, Tamoufe *et al.*, 2007) shows no CCR5-Δ32 mutants in a population of 1390 rural inhabitants. Similarly a study done on West and Central Africans also demonstrate an absence of CCR5-Δ32 gene in this population (Samson, Libert & Doranz, 1996).

Thus all studies point out to the absence or negligible frequency of the CCR5-Δ32 mutation in blacks or persons of African origin. In this study none of the participant was either white or coloured and further to that in Zimbabwe marriages between black Zimbabweans and whites are generally rare, may be this is the reason why this gene was not detected in this study population.

Although this study failed to detect any homozygous CCR5-Δ32 gene in Harare, the gene might not be totally absent from the Zimbabwean population, as it is believed to be present in the Lemba tribe. This follows DNA research that has traced the Lemba's origins to the Jews of the Middle East in Israel. Further to that a genetic marker largely found only in Cohanim, descendants of the ancient Jewish priesthood, is present in the same proportions among the Lemba's own priests, known as the Buba (The Zimbabwean situation, 2010). The CCR5-Δ32 gene has a prevalence rate of between 20-35% among the Jews (Spinney, 2003). Because of

that the CCR5-Δ32 gene might be present in the Lemba tribe of Zimbabwe, though this could not be detected in the current study because these people constitute less than 1% (80 000) of the Zimbabwean's total population and are found about 700 km away from the study area (The Zimbabweansituation, 2010).

5.2.3 Association between CCR264I and HIV infection

The current study re- affirm the suggestion that the distribution pattern of the rate and severity of the HIV epidemic may be indicative of greater underlying susceptibility to the virus in certain populations, which may be attributable to certain ubiquitous factors such as host genetics (Martinsoni, 1997). The reason for this is that the current study reports no protective traits in CCR2V64I gene and absent of CCR5-Δ32 in the study population. The current findings have added more controversy in the debate surrounding the impact of CCR2 on HIV infection, as CCR2V64I was associated with HIV infection (RR=2.321, 95% CI =1.376-3.916, p=0.002) in this study. Yet the majority of studies suggest that CCR2-64I, a conservative valine to isoleucine substitution in the transmembrane region, is associated with a significant delay in the progression to AIDS even in heterozygotes (Chakraborti & Banerjea, 2002; Kostrikis *et al.*, 1998; Mulherin, O'Brien, Ioannidis, Goedert, Buchbinder, Coutinho, 2003).

In agreement with the current study, Singh and colleagues (2009) found that (homozygous CCR264I) CCR2-A/A genotype was associated with higher risk of vertical transmission vs G allele (CCR2 wild type) carriers suggesting a modest effect of CCR2 genotypes on Mother-To- Child- Transmission of HIV in mother-infant pairs in sub-Saharan. Elsewhere, a study to assess the association between perinatal HIV transmission and genetic polymorphism of chemokine co-receptors by Lousirochanakul and co-workers (2004) report a higher prevalence of CCR2 V64I gene among infected children than in uninfected children (4% vs

1%) in Thailand. In contrast to that, Mangano and collaborators (1998) observed the protective effect of CCR2-A/A (CCR2V64I/64I) genotype in Argentinean children born to HIV-1 infected mothers. On the other hand, Teglas and co-workers (1999) and Brouwer and group (2005) failed to find any impact of the CCR2 genotype on perinatal transmission in France and Western Kenya, respectively.

The current study suggest that, although CCR2 V64I gene has been reported to delay progression of HIV to AIDS, (Chakraborti & Banerjea, 2002; Kostrikis *et al.*, 1998; Weissman *et al.*, 1997, Mulherin *et al.*, 2003; Christine, 2001), the gene may however, have some predisposal effect to HIV infection. A number of host genes have been observed to exhibit similar characteristics as currently shown by the CCR2 V64I gene. For instance the Duffy Antigen Receptor for Chemokines -46C/C (DARC-46C/C) is associated with 40% increase in the odds of acquiring HIV-1, but once an individual is infected with HIV, those with the mutation live on average 2 years longer than those without it (He, Neil, Kulkarni, Wright, Agan, Marconi *et al.*, 2008).

The study design could have also affected the current results. The current study was a cross-sectional study. In cross-sectional studies the determination of exposure and effect are made at the same time, as a result it is not easy to assess the reasons for associations shown. For instance a cross-sectional study, using a seroprevalent cohort rather than a cohort that followed individuals from the time of seroconversion, disputed the protective effect of CCR2V64I against HIV infection (Michael *et al.*, 1997) just like our current study. Yet the majority of cohort studies seem to suggest that CCR2V64I gene delay progression of HIV to AIDS (Chakraborti & Banerjea, 2002; Kostrikis *et al.*, 1998; Mulherin *et al.*, 2003). On the other hand, high prevalence of CCR2V64I gene might be attributed to the fact that HIV infected people with the gene live longer (Chakraborti & Banerjea, 2002; Kostrikis *et al.*,

1998; Mulherin *et al.*, 2003), than those without the gene, as a result cross-sectional studies are bound to investigate those who are surviving leading to a rise in the number of infected people with the gene.

5.2.4 Effect of confounders on the results

There is compelling evidence that links STIs to HIV infection. Individuals who are infected with STIs are at least two to five times more likely than uninfected individuals to acquire HIV infection if they are exposed to the virus through sexual contact. In addition to that, if an HIV-infected individual is also infected with another STIs, the person is more likely to transmit HIV through sexual contact than other HIV- infected persons (Munjoma, Kurewa, Mappingure, Mashavave, Chirenje, Rusakaniko *et al.*, 2010; Centre for Disease and Prevention, 1992; Wasserheit, 1992). Susceptibility to HIV infection in STIs infection occurs in two ways. Genital ulcers (syphilis, herpes, chancroid) result in the breaking of the genital tract lining or skin. These breaks create a portal of entry for HIV. In addition to this, inflammation resulting from genital ulcers or non-ulcerative STDs (trichomoniasis) increases the concentration of cells like CD4 -Tcells in genital secretions that can serve as targets for HIV (Centre for Disease and Prevention, 1992).

In agreement with this, this study demonstrated that STIs were associated with HIV infection. Because of that we had to adjust for the confounding effect of these STIs in order to authentically measure the effect of CCR2 and CCR5 mutants on HIV transmission in the study population. We had therefore to use multivariate logistic regression analysis to adjust for the effect of the STIs. However, even after adjusting for the confounding effect of STIs, CCR2V64I gene continued to be associated with HIV infection among these pregnant women.

5.2.5 Study Limitations

The current study used sera samples for DNA assays because this is what was available. Use of either plasma or whole blood could probably have given us better results, because the DNA content in some of the sera samples was very low. Because of this we ended up with a sample size of 242 instead of 288, though this was still within the statistical acceptable range of 138 to 288 participants calculated using a prevalence rate of 10-25% as observed by Matte and group (2001). Further to that, there may be other confounders like the nutritional status, immunological status and the number of sexual partners that could not be accounted for in this study.



CHAPTER 6: CONCLUSION

The current study demonstrated that the homozygous CCR2V64I gene and STIs were prevalent in HIV infected pregnant mothers and was also associated with HIV infection in these pregnant women in Harare. Further to that the study found out that the homozygous CCR5-Δ32 gene was absent in the same population. The study may imply that African pregnant women in Epworth, St Mary's and Seke North in Harare may be more vulnerable to HIV-1 infection as they lack the protective effect conferred by the mutant form of CCR2 and CCR5 mutants, which block HIV-1 entry into the host cells or delays the progression of HIV to AIDS. This finding further strengthens the observation that the absence of the protective gene CCR5-Δ32 and the observed predisposal effect of CCR2V64I genotype might have contributed to the accelerated spread of HIV in African populations (Schliekelman *et al.*, 2001; Sullivan *et al.*, 2001). The current finding is a cause for public health concern in that CCR2V64I has a high frequency (13% to 25 %) among the African populations (Christine *et al.*, 2001). May be this partially explains the reason why Zimbabwe is rated among the top five countries hardest hit by the HIV pandemic in the world (UNAIDS, 2008a).

CHAPTER 7: RECOMMENDATIONS

The observation that CCR2V64 gene is associated with HIV infection as well as the absent of CCR5-Δ32 gene in the studied population is a cause for public health concern. Because of this further studies with a larger sample size should be carried out in Mberengwa district where we find the Lemba tribe who are said to be the descendents of the Israel Jews (The Zimbabweansituation, 2010) known to harbour both CCR2V64 and CCR5-Δ32 genes (Spinney, 2003; Christine *et al.*, 2001). Further to that the proposed study should also encompass investigation on the DARC -46C/C gene that could have influenced the current findings. Conducting further studies is important because information on the host genes to HIV infection remains useful in the designing of genetic polymorph targeted therapeutic interventions as well as for vaccine development.



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APPENDICES

Appendix 1: Record of English consent form

RECORD OF INFORMED CONSENT

**Project title: HOST GENETIC FACTORS TO HIV INFECTION AMONG
PREGNANT WOMEN FROM HARARE, ZIMBABWE**

Date:

Interviewer: White Soko



University of Western Cape Student no: 2706564

Tel: 263 4700457-9/0912432752 Fax: 263 4253978

E-mail: whitesoko@yahoo.co.uk

Institution: National Institute of Health Research, Zimbabwe

Place at which the samples will be stored and tested: University of Zimbabwe's Medical school

Thank you for agreeing to allow me to store and use your sample on Host genetic factors to HIV infection among mother-baby pairs. What follows is an explanation of the purpose and

process of this study. You are asked to give your consent to me so that I can store and conduct laboratory analyses of your sample to determine the role of the host genes to HIV transmission.

1. Information about the researcher

I am White Soko a student at the SOPH, University of the Western Cape. As part of my Masters in Public Health, I am required to conduct a research for use for the write up of my mini-thesis. I will be focusing on the Role of Host genes on HIV transmission in pregnant women. I am accountable to Prof M. Chirenje *791631 ex 2452* or by e-mail at chirenje@uz-ucsf.co.zw

Here is some information to explain the purpose and usage of your samples.

2. Purpose of the study

The purpose of this study is to determine the frequency of chemokine core receptors, CCR2-V641 and CCR5 delta 32 genes and their association with HIV infection among pregnant mothers. The proposed study is expected to produce information on the key host genes that are involved in HIV transmission in the African population in Zimbabwe. Furthermore the information that is going to be produced will contribute to the understanding of the role of CCR2-V641 and CCR5 delta 32 host genes, in the dynamics, evolution of the epidemic, and in designing effective chemotherapeutic drugs and vaccines directed against the chemokine core receptor sites.

3. The data collection process

Five (5) mls of stored serum samples from pregnant mothers who participated in the main study entitled "PREVENTION OF MOTHER TO CHILD TRANSMISSION OF SEXUALLY

TRANSMITTED INFECTIONS” now called “Better health for the African Mother and child” who consented to have their samples stored and used in this sub-study looking at the Frequency of host genetic factors to HIV infection among pregnant from Harare, will be used in testing for the chemokine receptor genes (CCR2V641 and CCR5 Δ 32 genes). The HIV status of the samples will be drawn from the main register.

4. Anonymity of contributors

At all times, I will keep the source of the information confidential. To keep your information private, your samples will be labelled with a code/number that can only be traced back to your study clinic. I shall keep any other records of your participation locked away at all times, and destroy them after the data has been collected. Your name, where you live, and other personal information will be protected by the study clinic. When researchers are given your stored samples, they will not be given your personal information. The results of future tests will not be included in your health records. Every effort will be made to keep your personal information confidential, but we cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law.

5. Things that may affect your willingness to participate

There are few risks related to storing your samples. When tests are done on the stored samples there is a rare but possible risk to your privacy. It is possible that if others found out information about you that is learned from tests (such as information about your genes) it could cause you problems with your family (having a family member learn about a disease that may be passed on in families) or problems getting a job or insurance.

If there is anything that you would prefer not to be done on your sample, please feel free to say so. I will not be offended and there will be no negative consequences if you would prefer not to answer a question. I would appreciate your guidance should I do anything which you see as intrusive.

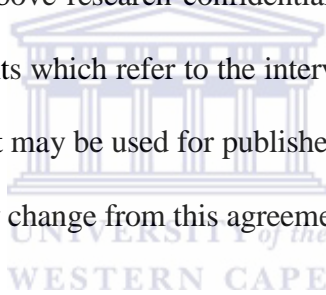
6. Agreement

6.1 Interviewee's agreement

The interviewee will be asked to give his/her consent below.

6.2 Interviewer's agreement

I shall keep the contents of the above research confidential in the sense that the codes noted above will be used in all documents which refer to the interview. The contents will be used for the purposes referred to above, but may be used for published or unpublished research at a later stage without further consent. Any change from this agreement will be renegotiated with you.



Signed by interviewer:

Signed by participant:

Date:

Place:

Whom to contact if you have problems or questions

For questions about the storage and testing of your samples, contact:

- *The National Coordinator*

Medical Research Council of Zimbabwe

National Institute of Health Research

Cnr Mazoe Street/ Josiah Tongogara Avenue, Harare

Ph +2634791792, 2634791193, Cell 263091433166

- *Principal Investigator UZ/UNO*

Obstetrics and Gynaecology

P.O Box A178

Avondale

Harare



Tel: 2634791631 ex 2452

Appendix 2: Record of consent form Shona version

RECORD OF INFORMED CONSENT

**Project title: HOST GENETIC FACTORS TO HIV INFECTION AMONG
PREGNANT WOMEN FROM HARARE, ZIMBABWE**

Date:

Researcher: White Soko

University of Western Cape Student no: 2706564

Tel: (04) 253975-9/ 0912432752

Fax: 263 4253978

E-mail: whitesoko@yahoo.co.uk

Institution: National Institute of Health Research, Zimbabwe

Place at which the samples will be stored and tested: University of Zimbabwe's Medical school

TSANANGUDZO YECHIRONGWA

Imi nemwana wenyu muri kukokwa kupinda muchirongwa chakanyorwa pamusoro chiri kuitwa neUniversity yedu yemuZimbabwe neyekuNorway chichitsigirwa neweLetten Foundation kuNorway zvakare. Kana mabvuma kupinda muchirongwa ichi, ropa rakatorwa muchirongwa chinonzi "PREVENTION OF MOTHER TO CHILD TRANSMISSION OF SEXUALLY TRANSMITTED INFECTIONS" chave kunzi "Better health for the African

Mother and child richa shandiswa kuongorora mahost genes anoti , CCR2-V641, ne CCR5 delata 32 genes. Tichawongorora wutachiwona hweHIV muropa iroro zve. Ropa rinoita mamiririta mashanu (5mls) rakatorwa kwamuri amai, nemwana wenyu rikachengetedzwa ndiro richashandiswa. Sezvo ropa rakakosha, muri kukumbirwa kuti ropa iri rizoshandiswazve mune zvimwe zvirongwa zvemangwana kana chironzwa ichi chaperu. Mune mibvunzo here pari zvino pane zvandatsanangudza. Kana mabvuma zvandatsanangudza pamusoro munoratidza nekusaina papepa rino.

KUPINDA MUCHIRONGWA HAKUMANIKIDZWE

Kubvuma kuti ropa renyu neremwana richengetedzwe hakumanikidzwi. Mune mvumo yekuti ropa renyu rigoshandiswe muchironzwa chino chete kana muchida uyezve mune mvumo yekuramba kuti ropa renyu rizoshandiswa mune zvimwe zvirongwa zvinotevera.

Kunyange zvazvo mabvuma iye zvino kuti ropa renyu ringachengeterwa kushandiswa mune zvimwe zvirongwa zvichatevera makasununguka kushandura pfungwa chero zvayo nguva. Kana izvi zvikaitika munofanira kuudza vashandi vechironzwa kuti mashandura pfungwa pamusoro peropa renyu rakachengetedzwa. Kana maramba kuti ropa renyu rizoshandiswa mune zvimwe zvirongwa zvichatevera rinoraswa panopera chironzwa chino.

DONZO RECHIRONGWA

Basa chairo rinoda kuitwa neropa renyu nderekuita ongororo dzechizvino-zvino. Ongororo yatirikuda kuita ndeyekuda kuona kuti pane zvinhu mumuviri yedu zvinonzi “mahost genes/HIV-1 chemokine receptor genes” zvino batsiridza kana kudzivirira kutapurirwana kwe hutachiwona hweHIV-1. Izvi zvinoitwa kuti muongororwe kuti mune mhando (yema host genes/HIV-1 chemokine receptor genes) ipi yemachemokine receptors. Tinoda zvakare

kuona kuti machemokines amai akafanana kana kusiyana sei nemwana wavo. Zvichabuda muongoro (maresults) zvichashandiswawo mukunyorwa kwegwaro (mini-thesis dissertation) reMasters in Public Health.

Hatina chinangwa chekukupai imi kana chiremba wenyu marizautsi eropa renyu rakachengetedzwa nokuti ongororo iyoyi haisati yakwenenzverwa zvinodiwa nemutemo.

Vaongorori vechirongwa havana urongwa hwekuzivisa imi kana chiremba wenyu nezviwanikwa zvetsvagurudzo ino. Izvi zvinoitwa nokuti ongororo iyi inoitwa nenzira isati yanyatsokwenenzverwa zvinotarisirwa zvekuti marizautsi anogona kusakubatsirai kuita sarudzo ine chokuita nekuchengetedza utano hwenyu. Apo tinowana zvingange zvakakosha zvine chokuita neutano hwenyu, vaongorori vanozozivisa vashandi vechirongwa avo vanozokuzivisaiwo. Kana muchida kuzozivisa nezviwanikwa zvakadai munofanira kuzivisa vashandi vechirongwa pamungazowanikwa. Kana muna chiremba wenyu wamunoda kuti azozivisa nezviwanikwa zvakadai munofanirawo kupa vashandi vechirongwa pavanokwanisa kuwanira chiremba wenyu.

Ropa renyu harizotengeswa kana kushandiswa kugadzira zvinowanisa mari.

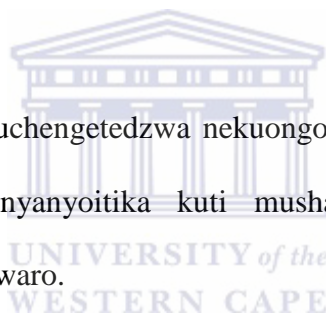
Mafambisirwo echirongwa achange achiongororwa newe Norwegian Ethical Review Committee pamwe chete nevanoona mafambisiro ezveongororo muZimbabwe, veMedical Research Council of Zimbabwe (MRCZ)

ZVICHAITWA

Ropa rinoita mamiririta mashanu (5mls) rakatorwa kwamuri amai, nemwana wenyu rikachengetedzwa pa-86°C kuUniversity yeZimbabwe department reObstetrics and Gynaecology, ndiro richashandiswa kuongorora “magemokine coreceptor genes”. Vanhu vari kushanda muchirongwa ichochi chete ndivo vanobvumirwa kushandisa ropa renyu uye patubhoro tweropa hatunyorwi zita renyu, asi panongoshandiswa nhamba chete. Naizvozvo vashandi ava havazivi zvachose kuti iri ropa nderaani. Ropa renyu ringangotumidzirwa kune vamwe vedu vatinoshandidzana nawo kuti riongororwe ikoko nemichina mitsva yemazuva ano yatisati tawanayo muno muZimbabwe.

NJODZI/ MARWADZO

Hapana njodzi inowanikwa mukuchengetedzwa nekuongororwa “magemokines coreceptor genes” kweropa renyu. Hazvinyanyoitika kuti mushandi arikuongorora ropa renyu rakachengetedzwa azive muridzi waro.



ZVINGAKUBATSIRAI MUCHIRONGWA

Hapana rubatsiro rwamunowana kubva muchirongwa chino kana kuchengetedzwa kweropa renyu. Asi zvichabuda muongororo zvichakubatsirai kuziva kuti mune mhando dzipi dzema chemokines receptor genes uye izvi zvakakosha mushishi yekutsvagwa kwemishonga yokudzivirira chirerwe cheHIV usati waonekwa pari zvino. Imi kana vamwewo vane denda irori vangangobatsirikana mune remangwana kubva mune zvichabuda muongororo iyoyi.

KUCHENGETEDZWA KWEZVICHABUDA MUONGORORO

Hapana zita renyu richashandiswa paongororo iyi asi tinoshandisa namba chete. Zita renyu, kero yenyu pamwe nezvimwe zvakabvunzwa pamusoro penyu zvichange zvakachengetedzwa. Vashandi wemuchirongwa vanoongorora ropa renyu havafi vakaziva zvinhu izvi. Zvinozobuda muzvirongwa zvemangwana hazvizonyorwi pakadhi renyu rekukiriniki. Zvinobvunzwa pamusoro peupenyu hwenyu , zita renyu nezvichabuda muongororo yeropa hazvifi zvakaudzwa chero munhu zvake kunze kwevashoma vanoshanda muchirongwa ichi .Vane mvumo yekuona zita renyu pamwe chete nezviri pamusoro penyu ndeweMedical Research Council of Zimbabwe (MRCZ) sezvinotarisirwa pamutemo neveNorwegian Ethical Committee.

Vavairo ichaitwa mukuedza kuchengetedza marekodzi pamwe nemarizautsi sezvinodiwa nemutemo.



SIGNATURE PAGE

CONSENT FOR SPECIMEN STORAGE AND SHIPMENT

Nyatsoverengai zvakanyorwa pasi kana musingakwanisi kuverenga ndinokuverengarai munyatsoita sarudzo yamunoda pamusoro peropa renyu neongororo dzichaitwa pariri. Kuramba kwenyu hakushandure mabafirwo amunoitwa muchirongwa chekutanga.

_____ Ndinobvuma kuti ropa rangu neremwana wangu richengetedzwe, uye rizoshandiswa muzvirongwa zvemangwana zvine chekuita nedenda reHIV pamwe chete ne chemokine coreceptor gene testing.

_____ Ndinobvuma kuti ropa rangu neremwana wangu richengetedzwe, uye rizoshandiswa muzvirongwa zvemangwana zvine chekuita nedenda reHIV. Asi

handibvumirani neongororo dzechemokine coreceptor gene testing kuti dziitwe paropa rangu neremwana wangu.

_____ Handibvumi kuti ropa rangu neremwana wangu richengetedzwe uye rizoshandiswa muzvirongwa zvemangwana zvine chekuita nedenda reHIV.

Participant Name (print)

Participant Signature or Mark and Date



Study Staff Conducting

Study Staff Signature and Date

Consent Discussion (print)

Witness Name (print)

Witness Signature and Date

(As appropriate)

MIBVUNZO

Kana mune mibvunzo yakanangana nekuchengetedzwa kweropa renyu, munogona kunyorera kana kuridza runhare kuna Prof M. Chirenje pakero inoti:

- Principal Investigator UZ/UNO

Obstetrics and Gynaecology

P.O Box A178

Avondale

Harare

Tel: 2634791631 ex 2452, email: chirenje@uz-ucsf.co.zw

- The National Coordinator

Medical Research Council of Zimbabwe
UNIVERSITY of the
WESTERN CAPE

National Institute of Health Research

Cnr Mazoe Street/ Josiah Tongogara Avenue

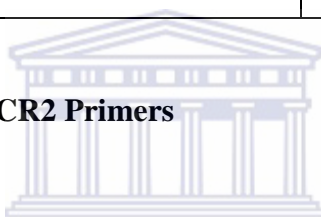
Harare

Ph 791792, 791193

Cell 091433166

Appendix 3: Specifications of CCR5 Primers

Primer	Sequence	Length	T _m	GC content
Sense Primer (Forward primer)	5'-TTCATTACACCTgCAgCTCTC	21-mer	54.0 ⁰ C	47.6%
Sense Primer (Reverse primer)	5'-gACCAgCCCAAAgATgACTATC	22-mer	57.6 ⁰ C	54.5%



Appendix 4: Specifications of CCR2 Primers

Primer	Sequence	Length	T _m	GC content
Sense Primer	5'-TTCATTACACCTgCAgCTCTC	20-mer	54.3 ⁰ C	50.0%
Antisense	5'-gACCAgCCCAAAgATgACTATC	20-mer	56.6 ⁰ C	50.0%

Appendix 5: Specifications of CCR2 Probes

Probe name	Sequence	Length	T _m	GC content
CCR2 Anchor	5'-LC640- CTgCAAAAAGCTgAAgTgCTTgACTg--PH	26-mer	64.3 ⁰ C	46.2%
CCR2-Sensor-mt	5'-AACATgCTggTCATCCTCATCTTAATAA- -FL	28-mer	59.2 ⁰ C	35.7%



Appendix 6: Preparation of 2% agarose gel for analysis of PCR products

1. Preparation of 1 % Agarose gel

- 2 g agarose is weighed and 100 ml of TAE (1x) is added
- Boil the mixture for a few minutes, swirling occasionally to prevent boil over, until all the agarose is dissolved.
- Let the solution cool to approximately 60.0°C (until it is cool enough to hold the flask in your hand)
- Add 1x SYBR safe solution
- Pour the gel and insert the comb
- After the gel has solidified the gel is put in the electrophoresis chamber filled with TAE (1x).
- Remove the comb; the gel is now ready to load your DNA samples on.

2. Loading of the gel

- Remove 5 µl of your PCR product, add to it 3µl loading dye and load the gel. One well is used for a marker containing DNA of known size and quantity (e.g 200bp)
- Electrophorese for approximately 1 hour at 80-100V . . After the electrophoresis the gel is submerged in a 0.5 to 1µg/ml Ethidiumbromide solution for about 20 to 30 minutes. The DNA is visualised by UV and a picture is taken.

3. Reagents

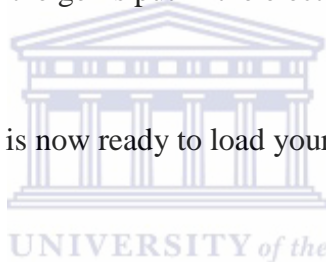
- Stock solution of TAE 50x:

2M Tris (242g)

1.66 M NaAc (136g)

0.1M EDTA (37.2g)

Stir till dissolved and add Acetic Acid up to a pH of 7.8. Add water up to 1 litre.



- Work solution of TAE 1x

Dilute the TAE (50x) stock solution 1/50 with deionised water to a final concentration of:

40mM Tris

33 mM NaAc

2mM EDTA

- Loading dye

40% sucrose

0.2 % Bromophenol blue



**Appendix 7: Procedure for the extraction of DNA from serum samples using the Boom
et al., (1999) method.**

Reagents

NucliSens Lysis Buffer

NucliSens isolation kit, NucliSens

Other Materials

Microcentrifuge

Centrifuge

Screw-cap 1.5 ml microtube

Dry heat temperature block

Sterile Transfer Pipettes

Dedicated fixes and variable pipettes tips

Vortex mixer

70% Ethanol

Acetone

Gloves



Method:

Thaw Lysis Buffer Wash Buffer until all crystals have dissolved (about 30 minutes).

Mix well.

1. Add 0.1 ml plasma to 0.9 ml of Lysis Buffer in labelled screw-cap microtubes.
Cap tightly and vortex briefly.
2. Vortex silica thoroughly. Add 50 μ l silica to each lysis tube, vortex and incubate 10 minutes at room temperature.
3. Quick spin lysis tubes, remove supernatant and 1,000 μ l of Wash Buffer. Vortex thoroughly and quick spin.

Repeat step 3

4. Remove supernatant and add 1,000 μ l of 70% Ethanol (made using 11 ml of 96% Ethanol and 4ml of distilled water). Vortex thoroughly and quick spin. Wash bench and change gloves.

Repeat step 4

5. Remove supernatant and add 1,000 μ l of Acetone. Vortex and quick spin. Remove ALL the acetone.
6. Dry silica/lysis tubes in a heat block at 56⁰ C caps off for 10 minutes. (You may cover tubes with a paper towel).
7. Add 50 μ l of Elution Buffer to each lysis/silica tube. Vortex
8. Incubate at 56⁰ C , caps on, for a total of 10 minutes, vortexing after 5 minutes.

9. Spin lysis/silica tubes for 2 minutes at maximum speed (10,000 x g).

For each specimen, remove 5 μ l of supernatant to fresh, labelled micro tube.

Avoid contaminating this aliquot with silica as silica can inhibit the amplification step. The rest of supernatant/silica should be kept at -70° C for up to a month.



Appendix 8: Standard procedure for real time PCR Amplification for CCR5 delta 32 using the Applied Biosystems Step One™ Plus Real-time PCR machine.

Reagents preparation

Stock Primers

Reconstitute your primers by dissolving 5nmol lyophilized primers into 0.5 mls of PCR grade distilled water in order to make a 10 nmol/ml stock solutions.

Dilution of stock solution to make a working solution of primers

Make a 1:5 dilution of stock solution of both the forward and reverse primers sufficient enough to use in the preparation of master mix.



Dilution of plasmids

Make a 1: 10 000 dilution working solution of plasmids from the stock solution.

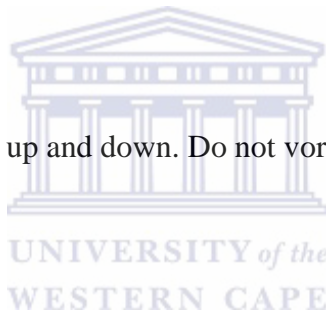
Set up of reaction for one sample

Reaction tube

- In a 1.5ml reaction tube on ice, prepare the PCR mix for one 21 µl reaction by adding the following components

Syber green master mix	-	10 μ l
D32-F2 Primers	-	3 μ l
D32-R Primers	-	3 μ l
Template/Sample	-	<u>5 μl</u> (to be added in the capillary tube)
Total volume	-	<u>21 μl</u>

- Prepare the PCR master mix for more than one reaction, by multiply the amount by in “volume” by column above by Z, where Z = the number of reactions (samples) to be run + one addition reaction/sample.



- Mix carefully by pipetting up and down. Do not vortex.

Micro well plate

- Pipet 15 μ l PCR mix into each micro 96 well plate.
- Add 5 μ l of CCR5- Δ 32 and CCR5 plasmids as well as distilled water in duplicates.
- Add 5 μ l of the DNA template into each micro plate well.
- Seal the micro plate with a parafilm.

- Centrifuge the micro plate briefly in a micro-centrifuge.

Amplification

1. Place the samples in a Applied Biosystems Step One Plus Real-time PCR machine to perform Forty cycles of amplification using the following temperature profiles:

Stage 1: Activation of enzyme

Step 1: 95⁰C for 10:0 minutes

Stage 2: PCRx 40 cycles

Step1: 95⁰C for 0:15s

Step 2: 60⁰C for 1:00 minutes

Stage 3: Melting curve

Step 1: 95⁰C for 0:15s

Step 2: 60⁰C for 1:00 minute, then 0.3⁰C/slope up to 95⁰C

Step 3: 95⁰C for 0:15s

Measure the fluorescence light at 520 nm using a fluoremeter. Amplicons containing the 32 –bp deletion are supposed to have a melting temperature (T_m) that is approximately 2⁰C lower than that of the 83 bp.

Appendix 9: Standard procedure for real time PCR Amplification for CCR2V64I on a LightCycler

REAGENTS PREPARATION

Primers and oligoprobes

1. Reconstitute primers by dissolving 5nmol in 0.5ml of PCR grade water to make a concentration of a 10 nmol/ml
2. Reconstitute the probes by dissolving 1mmol in 0.5 mmol of PCR grade water to make a concentration of 2nmol/ml

Set up of reaction for one sample

- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries. For volumes <20 μ l, the reaction and cycles conditions must be optimized.
- Depending on the total number of reactions, place the required number of LightCycler Capillaries in pre-cooled centrifuge adapters or in a LightCycler Sample Carousel in precooled LC Carousel Centrifuge Bucket.
- Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening. Mix carefully by pipetting up and down and store on ice.
- Prepare a 10 X conc. solution of PCR primers by and a 10 X conc. solution of HybProbe probes.

- In a 1.5 reaction tube on ice, prepare the PCR mix for one 20 μ l reaction by adding the following components

3mM MgCl₂ concentration master mix recipe for the master mix

PCR AMPLIFICATION MASTER MIX (for 25 μ l reaction volume)

Hyb Prob EnzymeMaster mix	-	2 μ l
MgCl ₂	-	1.6 μ l
CCR2-F Primers	-	2 μ l
CCR2-R Primers	-	2 μ l
CCR2 Sensor Probe	-	2 μ l
CCR2 Anchor Probe	-	2 μ l
DNATemplate	-	5 μ l
PCR Distilled Water	-	3.4 μ l
Total volume	-	<u>20 μl</u>



- Prepare the PCR master mix for more than one reaction, by multiply the amount by in “volume” by column above by Z, where Z = the number of reactions (samples) to be run + one addition reaction/sample.
- Mix carefully by pipetting up and down. Do not vortex.

- Centrifuge sample capillaries in a LC Carousel Centrifuge Bucket.

Amplification

3. Place the samples in a Light Cycler machine
4. Perform a 2 min denaturation at 95 °C.
5. Perform the 45 cycles of amplification using the following temperature profile:

Denaturation 95 °C, for 0 s

Primer annealing 64 °C for 15 s

Primer extension 72 °C for 25 s



6. Conduct a melting curve determination as follows:

(With a 0.2/sec slope from 45 °C to 95 °C)

95 °C for 20 s

64 °C for 25 s

58 °C for 25 s

7. Measure the fluorescence light using channels F2 (640nm) and F1 (520 nm) in a fluoremeter.



Appendix 10: Sequence of the CCR5 wt negative control plasmids



Gene Synthesis Quality Assurance Documentation

Müller 10/12-09
List in 1 and
TE-buffer
Bank ref - 1:10

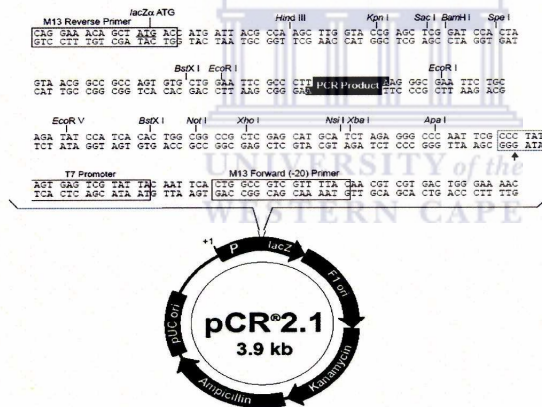
Order No.: 2055112/Müller

Plasmid Name:	pCR2.1-CCR5 wt	Internal Name:	C394-1
Gene Name:	CCR5 wt	Gene Size:	200bp
Vector Backbone:	pCR2.1	Antibiotic Selection:	Ampicillin and Kanamycin
Cloning:	cloned via TOPO-TA	Quantity:	5.9µg
Designation:	E. coli TOP10		

Genotype: F- mcr A Δ(mrr-hsdRMS-mcr BC) Φ 80lac ZΔM15 Δlac X74 rec A1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) end A1 nupG

Plasmid Map

PCR Product: CCR5 wt **5', 3' Cloning Sites:** none
cloned via TOPO-TA



Please Note:

Verify sequence after each cloning step. All material left at Eurofins MWG Operon will be discarded after 1 month.
Sequence analysis was done via doublestrand DNA sequencing. Sequence congruence was 100%.
Please find your sequence trace files in your personal account on our **ECOM system (order ID. 2055112)**.
The plasmid DNA has been lyophilised. We recommend to dissolve it in 10mM Tris buffer or TE.

Eurofins MWG Operon 10.12.2009

René Scheel
Project Manager Gene Synthesis

Sebastian Kubny
Project Assistant Gene Synthesis



Appendix 11: Sequence of CCR5 Δ 32 positive control plasmids



Müller 10/12-09
 List in 10ml TE-
 buffer
 Brück uf -1:10

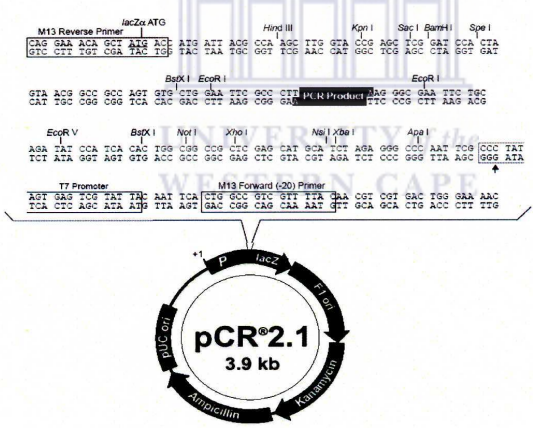
Gene Synthesis Quality Assurance Documentation

Order No.: 2055112/Müller

Plasmid Name:	pCR2.1-CCR5 d32	Internal Name:	C395-1
Gene Name:	CCR5 d32	Gene Size:	168bp
Vector Backbone:	pCR2.1	Antibiotic Selection:	Ampicillin and Kanamycin
Cloning:	cloned via TOPO-TA	Quantity:	8.4µg
Designation:	E. coli TOP10		
Genotype:	F- <i>mcr</i> A Δ (<i>mrr</i> - <i>hsd</i> RMS- <i>mcr</i> BC) Φ 80 <i>lac</i> Z Δ M15 Δ <i>lac</i> X74 <i>rec</i> A1 <i>ara</i> D139 Δ (<i>ara-leu</i>)7697 <i>gal</i> U <i>gal</i> K <i>rps</i> L (Str ^R) <i>end</i> A1 <i>nup</i> G		

Plasmid Map

PCR Product: CCR5 d32 5', 3' Cloning Sites: none
 cloned via TOPO-TA



Please Note:
 Verify sequence after each cloning step. All material left at Eurofins MWG Operon will be discarded after 1 month.
 Sequence analysis was done via doublestrand DNA sequencing. Sequence congruence was 100%.
 Please find your sequence trace files in your personal account on our **ECOM system (order ID. 2055112)**.
 The plasmid DNA has been lyophilised. We recommend to dissolve it in 10mM Tris buffer or TE.

Eurofins MWG Operon 10.12.2009

René Scheel
 Project Manager Gene Synthesis

Sebastian Kubny
 Project Assistant Gene Synthesis



Appendix 12: Sequence of CCR2 64V negative control plasmids



Mollath 19/12-09
Lohi 1ml TE-
buffer

Gene Synthesis Quality Assurance Documentation

Order No.: 2055112/Müller

Plasmid Name:	pCR2.1-CCR2 64V	Internal Name:	C393-3
Gene Name:	CCR2 64V	Gene Size:	380bp
Vector Backbone:	pCR2.1	Antibiotic Selection:	Ampicillin and Kanamycin
Cloning:	cloned via TOPO-TA	Quantity:	8.0µg
Designation:	E. coli TOP10		
Genotype:	F- <i>mcr</i> A Δ(<i>mrr</i> - <i>hsd</i> RMS- <i>mcr</i> BC) Φ 80 <i>lac</i> ZΔM15 Δ <i>lac</i> X74 <i>rec</i> A1 <i>ara</i> D139 Δ(<i>ara-leu</i>)7697 <i>gal</i> U <i>gal</i> K <i>rps</i> L (Str ^R) <i>end</i> A1 <i>mup</i> G		

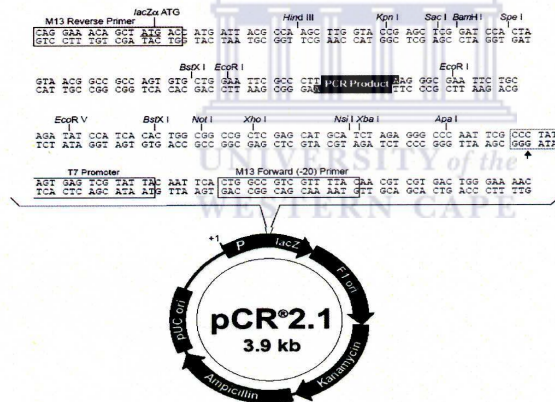
Plasmid Map

PCR Product:

CCR2 64V

5', 3' Cloning Sites: none

cloned via TOPO-TA



Please Note:

Verify sequence after each cloning step. All material left at Eurofins MWG Operon will be discarded after 1 month.

Sequence analysis was done via doublestrand DNA sequencing. Sequence congruence was 100%.

Please find your sequence trace files in your personal account on our **ECOM system (order ID. 2055112)**.

The plasmid DNA has been lyophilised. We recommend to dissolve it in 10mM Tris buffer or TE.

Eurofins MWG Operon 10.12.2009

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Sebastian Kubny
Project Assistant Gene Synthesis



Appendix 13: Sequence of CCR2 64I positive control plasmids for CCR2V64I gene

Müller 10/12-09
Lobi 1ml TE -
buffer and ~~test~~



Gene Synthesis Quality Assurance Documentation

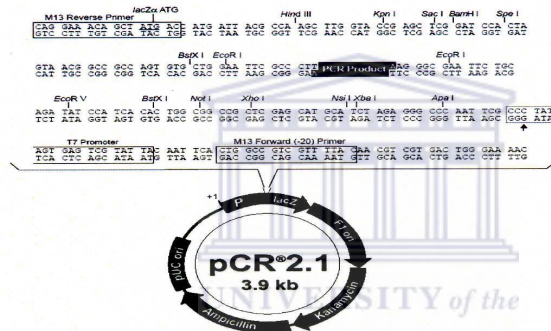
Order No.: 2055112/Müller

Plasmid Name:	pCR2.1-CCR2 64I	Internal Name:	C392-2
Gene Name:	CCR2 64I	Gene Size:	380bp
Vector Backbone:	pCR2.1	Antibiotic Selection:	Ampicillin and Kanamycin
Cloning:	cloned via TOPO-TA	Quantity:	8.4µg
Designation:	E. coli TOP10		
Genotype:	F- <i>mcr A</i> Δ(<i>mrr-hsdRMS-mcr BC</i>) Φ 80 <i>lac Z</i> Δ <i>M15 lac X74 rec A1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>gal U gal K rps L</i> (Str ^R) <i>end A1 nup G</i>		

Plasmid Map
PCR Product:

CCR2 64I

5', 3' Cloning Sites: none
cloned via TOPO-TA



Please Note:

Verify sequence after each cloning step. All material left at Eurofins MWG Operon will be discarded after 1 month.
Sequence analysis was done via doublestrand DNA sequencing. Sequence congruence was 100%.
Please find your sequence trace files in your personal account on our **ECOM system (order ID.2055112)**.
The plasmid DNA has been lyophilised. We recommend to dissolve it in 10mM Tris buffer or TE.

Eurofins MWG Operon 10.12.2009

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